qPCR 2009 9 – 13th March 2009

Symposium & Exhibition & Workshops

Main topics: *Diagnostics & Molecular Markers*

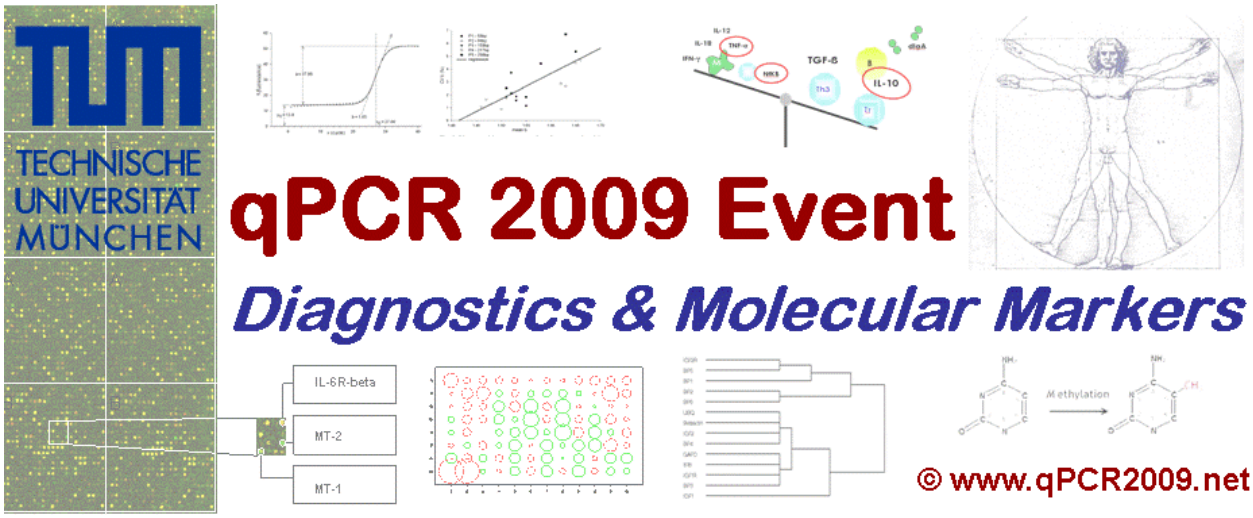
4th int.qPCR Event, Technische Universität München, Freising-Weihenstephan, Germany

Online Proceedings

qPCR 2009 Event

4th International qPCR Symposium
Industrial Exhibition & Application Workshops

Diagnostics & Molecular Markers



Editor: Michael W. Pfaffl

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Technical University Munich (TUM)
85354 Freising, Germany

www.qPCR2009.net

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Invitation

Dear colleagues,
Dear researchers,
Dear company representatives,

On behalf of the organisation committee and the scientific board of the conference it is a great pleasure to invite you to the qPCR 2009 Event, the 4th International qPCR Symposium, including an Industrial Exhibition and four qPCR Application Workshops. The symposium focus is **Diagnostics & Molecular Markers** and 86 lectures and 147 posters will be presented by international recognised experts in their application fields. The emphasis will be on unbiased, didactic information exchange. One third of the talks will be presented by invited speakers, one third of the speakers will be selected from the submitted abstracts and one third will be qPCR company representatives. Various poster sessions will be presented in parallel in a separate poster exhibition hall. All scientific contributions are fully citable by this qPCR 2009 Symposium Proceedings (ISBN 9783000268267).
It is a pleasure to announce the **Nobel Prize Laureate Kary Mullis** in an own plenary talk on Monday afternoon with the title ***"25th Anniversary of PCR"***

The qPCR Event is structured in three parts:

1. **qPCR Symposium** taking place March 9-11, including various Talk and Poster Sessions;
2. A parallel **qPCR Industrial Exhibition** taking place March 9-11;
3. Followed by four **qPCR Workshops** taking place March 12-13, powered by BioEPS GmbH and the TATAA Biocenter Germany

The scientific organization is managed by international well-known scientists in the field of real-time PCR:

Stephen Bustin	Prof. of Molecular Science, School of Medicine, London, UK
Mikael Kubista	Prof. of Biotechnology, TATAA Biocenter, Sweden
Jo Vandesompele	Prof. at the Center of Medical Genetics, University of Ghent, Belgium
Heinrich H. D. Meyer	Prof. of Physiology, Technical University of Munich, Weihenstephan, Germany
Michael W. Pfaffl	Senior Scientist and Reader in Physiology, TUM Weihenstephan, Germany (Scientific coordination)

The event organization will be managed by Dr. Martina Reiter, BioEPS GmbH Martina.Reiter@bioEPS.com

The event location is the central lecture hall complex and the foyer at TUM (Technical University of Munich) in Freising Weihenstephan, Germany. The TUM and the Biotech region around Munich are part of the largest Biotech cluster in Europe, located close to the Munich airport (MUC) directly in the heart of Bavaria.

Please enjoy the conference, the exhibition, the workshops and the social program during the following week.

Best regards

Scientific coordinator

qPCR 2009 - Talk and Poster Sessions:

http://sessions.qpcr2009.net/

Main topic: Diagnostics & Molecular Markers
Markers in diagnostic, prognostic, and therapeutic, markers on DNA, RNA, microRNA, protein, and metabolite level, disease markers, tissue specific markers, cancer markers, stem-cells markers, differentiation markers, methylation markers, diagnostic quantification methods, epigenetics, SNP analysis, HRM = high resolution melt applications, development, establishment, optimization of immuno-qPCR, innovative immuno qPCR applications, …..

Main topic: Diagnostics & Molecular Markers in agricultural and veterinary Science
Diagnostics & Molecular Markers in White-, Green-, Blue-, and Yellow- Biotechnology and in agricultural and veterinary Science. Marker genes on diagnostic, prognostic, and therapeutic markers on DNA, RNA, microRNA, protein, and metabolite level, in animals and plants, … …

"25th Anniversary of PCR" Session held by Nobel Prize Laureate Kary Mullis

Single-cell qPCR
single-cell sampling, pre-amplification techniques, laser micro dissection, sub-cellular PCR, micro-manipulation of cell clusters, cellular micro injection, FACS spotting, single cell handling, ……

RNAi - microRNA - siRNA Applications
RNAi mechanism, microRNA extraction, qRT-PCR technologies to detect microRNA, siRNA applications in combination with qRT-PCR, microRNA targets and microRNA precursors, new siRNA manipulation and microRNA technologies, ...

High throughput quantitative PCR
384 well applications, new high throughput platforms, qPCR robotics, digital PCR, SNP application, gene expression real-time RT-PCR arrays (mRNA and microRNA), quantitative multiplexing,

qPCR NOS Session - Normalization & Optimization & Standardization
new types of normalization, one vs. multiple reference genes, genomic DNA as standard, external standards, optimization of the real-time PCR, inhibition of negative effects, optimization of real-time PCR efficiency, qPCR robotics, multiplexing, establishment of DNA / RNA standards, inter-run standards, national and international studies on qPCR standardization, new quantification strategies, Pre-amplification, sampling technologies, DNA / RNA purification, extraction efficiency, DNA / mRNA / microRNA quality control, Reverse Transcription, RT quality control, external references, …..

qPCR BioStatistics & BioInformatics
software applications, data mining, calculation of relative expression, primer and probe design on mRNA and microRNA level, real-time PCR efficiency determination, mathematical modelling, Multivariate expression profiling raw data analysis, statistics in real-time PCR, data management, multi-way expression profiling, multiple regression analysis, 3D data visualization, ...

qPCR 2009 Event Agenda

online agenda => http://online-agenda.qPCR2009.net/

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Seminar rooms S1 & S2</th></tr>
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8th Mach 2009</td><td rowspan="2"></td><td rowspan="2"></td><td rowspan="2"></td><td>13:00 – 18:00
Industrial Exhibition Built up</td></tr>
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Arrival & Registration</td></tr>
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Welcome by
Michael W. Pfaffl &
Wolfgang A. Herrmann
President TUM</td><td></td><td>9:00 – 12:00
Poster Setup</td><td>8:00 – 10:00
Arrival & Registration</td></tr>
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Diagnostic & Molecular Markers Session (1)</td><td></td><td>10:00 – 15:00
Poster Session</td><td rowspan="4">10:00 – 22:00
Industrial Exhibition</td></tr>
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Lunch</td></tr>
<tr><td>13:30 – 16:00
Diagnostic & Molecular Markers Session (2)</td><td>13:00 – 16:00
High Resolution Melting & Genotyping Session</td><td>10:00 – 15:00
Poster Session</td></tr>
<tr><td colspan="3">16:30 – 18:00
Nobel Prize Laureate Lecture by K. Mullis:
25th Anniversary of PCR</td></tr>
<tr><td colspan="4">18:00 – 22:00 Get Together Party
in the Foyer / Industrial Exhibition</td></tr>
<tr><td rowspan="4">Tuesday

10th March 2009</td><td>8:30 – 12:30
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Poster Session</td><td>8:30 – 19:00
Industrial Exhibition</td></tr>
<tr><td colspan="3">12:30 – 13:30
Lunch</td></tr>
<tr><td rowspan="2">13:30 – 18:30
Diagnostics & Molecular Markers in agricultural and veterinary Science</td><td>13:30 – 16:00
Single Cells Session</td><td rowspan="2">10:00 – 15:00
Poster Session</td><td rowspan="2">8:30 – 19:00
Industrial Exhibition</td></tr>
<tr><td>16:30 – 18:30
TUTORIALS in Biostatistics & Bioinformatics</td></tr>
<tr><td colspan="5">19:00 – 24:00 Symposium Gala Dinner
Location: Lindenkeller, Pasta & More, Freising
International Buffet, Asian Buffet, Music & Dancing</td></tr>
<tr><td rowspan="4">Wednesday

11th March 2009</td><td>8:30 – 12:30
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qPCR NOS Session (1)</td><td>10:00 – 13:00
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<tr><td colspan="3">12:30 – 13:30
Lunch</td></tr>
<tr><td>13:30 – 17:30
Biostatistics & Bioinformatics</td><td>13:30 – 17:00
qPCR NOS Session (2)</td><td>13:00 – 15:00
Poster Take Down</td></tr>
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Closing of the Symposium
Heinrich HD. Meyer & Michael W. Pfaffl</td></tr>
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Agenda qPCR 2009 Event

Sunday 8th March 2009

13:00 – 18:00 Built-up for Industrial Exhibition

15:00 – 18:00 Arrival & Registration

Monday 9th March 2009

Welcome & Opening of the Symposium
Lecture hall HS 14

08:00 – 10:00 Built-up for Industrial Exhibition

08:00 – 10:00 Arrival & Registration

09:00 – 10:00 **Welcome Coffee & Tea**

10:00 Welcome & Opening of the Symposium.
Michael W. Pfaffl
Scientific coordination of the qPCR 2009 Symposium

10:15 Welcome at the Center of Food & Life Science in Freising Weihenstephan.
Prof. Wolfgang A. Herrmann
President TUM, Germany

HOT TOPIC - KEYNOTE LECTURE

10:30 **MIQE- guidelines for publication of qPCR data**
Stephen A Bustin[1], Vladimir Benes[2], Jeremy A Garson[3], Jan Hellemans[4], Jim Huggett[3], Mikael Kubista[5], Reinhold Mueller[6], Tania Nolan[7], Michael W Pfaffl[8], Gregory L Shipley[9], Jo Vandesompele[4] and Carl T Wittwer[10]
[1]Barts and the London School of Medicine, UK; [2]Genomics Core Facility, EMBL Heidelberg, Germany; [3]University College London, UK and UCL Hospitals NHS Foundation Trust, UK; [4]Ghent University Hospital, Belgium; [5]Institute of Biotechnology AS CR, Czech Republic and TATAA Biocenter, Sweden; [6]Sequenom, USA; [7]Sigma-Aldrich, UK; [8]Technical University Munich, Germany; [9]University of Texas Health Science Centre, USA; [10]University of Utah USA and ARUP Institute for Clinical and Experimental Pathology, USA; s.a.bustin@qmul.ac.uk

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Session Diagnostic & Molecular Markers (1)
Chair J. Huggett & MW. Pfaffl
Lecture hall HS 14

Session sponsored by:

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Real-time PCR solutions

11:00 **A Multi-Assay Approach to the Study of Cellular Toxicity**
Gregory L Shipley
UTHSC-Houston, United States of America;
gregory.l.shipley@uth.tmc.edu

11:30 **Circulating nucleic acids in melanoma diagnosis**
Pamela Pinzani[1], Francesca Salvianti[1], Roberta Cascella[1], Vincenzo De Giorgi[2], Daniela Massi[3], Mario Pazzagli[1] and Claudio Orlando[1]
[1]Department of Clinical Physiopathology, University of Florence, Florence, Italy; [2]Department of Dermatological Sciences, [3]Department of Human Pathology and Oncology, University of Florence, Florence, Italy;
p.pinzani@dfc.unifi.it

12:00 **Prognostic multigene expression classification of cancer patients: a route for success**
Joëlle Vermeulen[1], Katleen De Preter[1], Filip Pattyn[1], Liesbeth Vercruysse[1], Nurten Yigit[1], Jan Hellemans[2], Frank Speleman[1] and Jo Vandesompele[2]
[1]Center for Medical Genetics, Ghent University Hospital, Belgium; [2]Ghent University, Belgium - Biogazelle, Belgium; joke.vandesompele@ugent.be

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12:30 – 13:30 **Lunch in the student cafeteria**

Session Diagnostic & Molecular Markers Session part 2
Chair S. Bustin & G. Shipley
Lecture hall HS 14

13:30 **A Novel Multiplex, Quantitative Gene Expression Approach for Cancer Biomarker Research**
Jim Thorn
Beckman Coulter UK, UK, JTHORN@beckman.com

14:00 **The use of nucleic acid amplification tests for research and diagnosis in the developing world.**
Jim Francis Huggett[1], Clare Green[1], Michael Hoelscher[2] and Alimuddin Zumla[1]
[1]Centre for Infectious Diseases and International Health, University College London, UK; [2]Department of Infectious Diseases and Tropical Medicine, Klinikum of the University of Munich, Germany; j.huggett@ucl.ac.uk

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14:30 **Effective placement of LNA into Q-PCR Probes**
Raymond Peterson
Celadon Laboratories, United States of America;
acollins@celadonlabs.com

15:00 **Expression signatures in IBD classification: A new approach**
Petra von Stein
InDex Pharmaceuticals AB, Sweden;
petra.stein@indexdiag.com

15:30 **Intragraft expression profiles by quantitative PCR in kidney transplant patients reflect variability in the response to anti-rejection treatment with corticosteroids**
Niels Rekers[1], Ingeborg Bajema[2], Kim Zuidwijk[3], Marko Mallat[3], Natascha Goemaere[4], Marian van Groningen[2], Cees van Kooten[3], Hans de Fijter[3], Frans Claas[1] and Michael Eikmans[1] [1]Department of Immunohematology and Bloodtransfusion, [2]Department of Pathology, [3]Department of Nephrology, Leiden University Medical Center, The Netherlands; [4]St. Pathan, Rotterdam, The Netherlands; n.v.rekers@lumc.nl

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16:00 – 16:30 **Coffee break**

Session Nobel Prize Laureate Lecture
Chair R. Cook & MW. Pfaffl
Lecture hall HS 14 16:30 – 18:00

Nobel Prize Laureate Kary Mullis:
25th Anniversary of PCR

Session sponsored by:

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18:00 – 22:00 **Get Together Party**

Session High Resolution Melting & Genotyping
Chair J. Hellemans & A. Stahlberg
Lecture hall HS 15

13:00 **Applications of HRM curve analysis: strengths and pitfalls.**
Kim De Leeneer, Ilse Coene, Bruce Poppe, Anne De Paepe and Kathleen Claes
CMGG, Belgium; kim.deleeneer@Ugent.be

13:30 **Using Melt Curve Analyses for Experimental Inquiry**
Madeline O'Donoghue, Junko Stephens, Nathalie Koch, Jonathan Wang, Gordon Janaway, Laurel Nelson
Applied Biosystems – part of Life Technologies, Foster City, CA madeline.odonoghue@appliedbiosystems.com

14:00 **Probe Based Detection of Genetic Variations - Screening and in-vitro Diagnostics**
Olfert Landt
TIB Molbiol GmbH, Germany; olandt@tib-molbiol.de

14:30 **Releasing the potential of High Resolution Melting analysis**
Rob Powell
PrimerDesign Ltd, United Kingdom;
rob@primerdesign.co.uk

15:00 **Simultaneous Determination of SNP Genotype and Allelic Copy Number of DME Gene CYP2D6**
Adam Broomer, Toni Ceccardi, Kelly Li, Yu Wang, Chunlin Xiao and Caifu Chen
Life Technologies, United States of America;
chencx@appliedbiosystems.com

15:30 **Haematopoietic Chimerism Analysis after Allogeneic Stem Cell Transplantation.**
Rosalind Ganderton[1], Kate Parratt[2], Deborah Richardson[2], Kim Orchard[2] and Elizabeth Hodges[1]
[1]Department of Molecular Pathology, Southampton University Hospitals NHS Trust, Southampton, United Kingdom; [2]Department of Haematology, Southampton University Hospitals NHS Trust, Southampton, United Kingdom; rhg@soton.ac.uk

16:00 – 16:30 **Coffee break**

Session Nobel Prize Laureate Lecture
Chair R. Cook & MW. Pfaffl
Lecture hall HS 14 16:30 – 18:00

Nobel Prize Laureate K. Mullis: *25th Anniversary of PCR*

Session sponsored by:

BIOSEARCH TECHNOLOGIES

18:00 – 22:00 **Get Together Party**
in the Foyer / Industrial Exhibition

Weihenstephan
ÄLTESTE BRAUEREI DER WELT

Tuesday 10th March 2009

Session Diagnostic & Molecular Markers (3)
Chair U. Reischl & H. Nitschko
Lecture hall HS 14

Session sponsored by:

PrimerDesign
Real-time PCR solutions

8:30 **Current applications of real-time PCR technology in diagnostic bacteriology**
Udo Reischl
University Hospital of Regensburg, Germany;
udo.reischl@klinik.uni-regensburg.de

9:00 **Real-time PCR Applications in the diagnostic of highly pathogenic viruses**
Andreas Nitsche
Robert Koch Institute, Germany; nitschea@rki.de

9:25 **Multiplex-PCR in clinical virology - benefits and limitations**
Hans Nitschko, Helga Mairhofer and Anna-Lena Winkler
Max von Pettenkofer-Institute, Germany;
nitschko@mvp.uni-muenchen.de

9:50 **Realtime PCR of bioterrorism agents**
Dimitrios Frangoulidis and Hermann Meyer
Bundeswehr, Germany;
DimitriosFrangoulidis@Bundeswehr.org

10:15 – 10:45 **Coffee break**

10:45 **Trans-renal DNA for infectious disease diagnosis**
Clare Green, Jim Huggett and Alimuddin Zumla
Centre for Infectious Diseases & International Health, University College London, United Kingdom;
clare.green@ucl.ac.uk

11:10 **New probes, same procedure - Improved results.**
Remove the false negatives and positives.
Ulf Bech Christensen
PentaBase, Denmark; ubc@pentabase.com

11:35 **The use of DNA/RNA chimeric primers in qPCR for microbial detection and quantification**
Ofer Peleg[1], Gad Baneth[2], Osnat Eyal[2], Jakob Inbar[1] and Shimon Harrus[2]
[1]Genaphora Ltd, Israel; [2]Koret School of Veterinary Medicine, The Hebrew University of Jerusalem;
ofer.peleg@gmail.com

12:00 **Prevalence and viral load of oncogenic Human Papillomavirus types associated with cervical carcinoma in a population of North Italy**
Francesco Broccolo
University of Milan-Bicocca, Italy;
francesco.broccolo@unimib.it

12:30 – 13:30 **Lunch in the student cafeteria**

Tuesday 10th March 2009

Session Diagnostics & Molecular Markers in agricultural & veterinary Science
Chair HHD. Meyer & U. Busch
Lecture hall HS 14

13:00 **Rapid detection and differentiation of Campylobacter jejuni, C. coli and C. lari in food samples using a quadruplex real-time PCR assay**
Anja Mayr[1], Johann Bauer[2], Diana Thäringen[1], Ulrich Busch[1] and Ingrid Huber[1]
[1]Bavarian Health and Food Safety Authority, Germany; [2]Institute of Animal Hygiene, TUM;
Ingrid.Huber@lgl.bayern.de

13:25 **The use of transcriptomics for biomarker development to trace anabolic hormone functions.**
Irmgard Riedmaier, Christiane Becker, Michael W Pfaffl and Heinrich HD Meyer
Technical University Munich, Germany;
irmgard.riedmaier@wzw.tum.de

13:50 **Development of a highly sensitive and specific assay to detect Staphylococcus aureus in bovine mastitic milk**
Hans Ulrich Graber
University of Bern, Switzerland;
hans.graber@knp.unibe.ch

14:15 **Development of a real-time PCR Method for Detection and Quantification of the Fungal Biocontrol Agent Trichoderma atroviride SC1 in Soil**
Federica Savazzini[1], Claudia Longa[2] and Ilaria Pertot[2]
[1]CNR, Istituto di Biologia e Biotecnologia Agraria, Via E. Bassini 15, 20133 Milano, Italy; [2]FEM-IASMA, Department of Plant Protection, Via Mach 1, 38010, San Michele all'Adige, TN, Italy; savazzinif@yahoo.com

14:40 **Temperature influence on expression of selected genes in Mycobacterium avium subsp. paratuberculosis in milk environment**
Radka Pribylova, Kralik Petr, Michal Slany and Ivo Pavlik
Veterinary Research Institute, Czech Republic;
slany@vri.cz

15:05 **Applying Real-time PCR to Determine Co-dominant Genotypes of Dominant SCAR Markers in Common Bean**
George J Vandemark[1], Phillip N Miklas[1], Deidre Fourie[2] and Richard Larsen[1]
[1]USDA ARS, United States of America; [2]ARC Grain Crops Institute, Potchefstroom, South Africa;
george.vandemark@ars.usda.gov

15:30 – 16:00 **Coffee break**

16:00 **Comparison of AOE activities and expression levels in the kidney during the development of hypertension in SHR**
S Arunkumar[1], SK Lee[1], KNS Sirajudeen[2] and HJ Singh[3]
[1]Department of Physiology, School of Medical Sciences, University Science Malaysia, Health Campus, 16150 Kubang Kerian, Kelantan, Malaysia.; [2]Department of Chemical Pathology, School of Medical Sciences, University Science Malaysia, Health Campus, 16150 Kubang Kerian, Kelantan, Malaysia.; [3]Faculty of Medicine, Universiti Technologi MARA, 40450 Shah Alam, Selangor, Malaysia.; vsbarun@yahoo.com

16:25 **A quantitative real-time PCR assay for Ehrlichia ruminantium using pCS20**
Helena Steyn
ARC-Onderstepoort Veterinary Institute, South Africa;
steynh@arc.agric.za

16:50 **Relative gene expression of acid-inducible genes in acid-adapted Escherichia coli O157:H7 during lactoperoxidase and lactic acid challenge**
ANGELA PARRY-HANSON[1], PIET JOOSTE[2] and ELNA BUYS[1]
[1]UNIVERSITY OF PRETORIA, South Africa; [2]TSHWANE UNIVERSITY OF TECHNOLOGY, South Africa; angiep@tuks.co.za

17:15 **A QUANTITATIVE PCR TECHNIQUE FOR EVALUATION OF ARSENIC MOBILITY IN HEAVY METAL CONTAMINATED SAMPLES**
Elena A. Polishchuk, Jie Chen, Vivian Lai and William R. Cullen
University of British Columbia, Canada;
elena@chem.ubc.ca

17:40 **Use of real-time PCR for detection of bovine herpesvirus-1 in cattle and buffalo frozen semen**
Samir Kumar Rana[1], Srinivasan Alwar Villuppanoor[2], Sri Naga Leela Surendra Kota[2], Sriraman Rajan[2] and Penchala Narasimha Rao Samyam[2]
[1]National Dairy Development Board, C/O Indian Immunologicals Ltd., Gachibowli, Hyderabad 500 032, India; [2]Indian Immunologicals Ltd., Gachibowli, Hyderabad 500 032, India; skrana@indimmune.com

18:05 **Viability of Mycobacterium avium subsp. Para-tuberculosis as measured by PMA-F57real time qPCR**
Petr Kralik and Ivo Pavlik
Veterinary Research Institute, Czech Republic;
kralik@vri.cz

19:00 – 24:00 **Symposium Gala Dinner**

Location: Lindenkeller Pasta & More, Freising
International Buffet, Asian Buffet, Music and Dancing

Pasta & more

Tuesday 10th March 2009

Session RNAi: microRNA – siRNA Applications
Chair M. Castoldi & MW. Pfaffl
Lecture hall HS 15

Session sponsored by.

IMGM® LABORATORIES

8:30 **miQPCR: A novel approach for expression profiling of mature microRNAs.**
Mirco Castoldi
EMBL, Germany; castoldi@embl.de

9:00 **An inflammatory microRNA signature in muscle cells - a comparative study of cellular models and technological platforms.**
Swanhild Meyer[2], Carola Wagner[3], Michael W Pfaffl[2] and Christian Thirion[1,4]
[1]SIRION BIOTECH, Martinsried, Germany; [2]Lehrstuhl für Physiologie, TUM München, Freising-Weihenstephan, Germany; [3]IMGM Laboratories, Martinsried, Germany; [4]Laboratory for molecular Myology, Friedrich-Baur-Institute, Department of neurology LMU Munich, Munich, Germany; Thirion@sirion-biotech.de

9:25 **Discovery and Validation of Novel Human MicroRNA Genes by SOLiD(TM) and TaqMan®**
Jason H. Halsey
Life Technologies / Applied Biosystems, United States of America; halseyjh@appliedbiosystems.com

9:50 **mRNA & microRNA integrity - the key to success**
Michael W Pfaffl, Christiane Becker, Andrea Hammerle-Fickinger and Irmgard Riedmaier
TUM, Physiology, Weihenstephan, Germany; michael.pfaffl@wzw.tum.de

10:15 **microRNAs - developing new tools for diagnostics - Join forces with IMGM Laboratories to make your miRNA project a success**
Carola Wagner
IMGM Laboratories GmbH, Martinsried, Germany; carola.wagner@imgm.com

10:40 – 11:00 **Coffee break**

11:00 **A novel and universal method for microRNA RT-qPCR data normalization**
Pieter Mestdagh[1], Pieter Van Vlierberghe[1], An De Weer[1], Frank Speleman[1] and Jo Vandesompele[2]
[1]Center for Medical Genetics, Ghent University Hospital, Belgium; [2]Ghent University, Belgium - Biogazelle, Belgium; joke.vandesompele@ugent.be

11:25 **Highly sensitive and specific LNA™-enhanced real-time PCR for microRNA expression analysis**
Ditte Andreasen, Nana Jacobsen, Liselotte Kahns, Kim Bundvig Barken, Rolf Søkilde and Peter Mouritzen
Exiqon, Denmark; dia@exiqon.com

11:50 **Quantification and Functional Analysis of miRNA in Mammalian Cells**
Martin Kreutz
QIAGEN GmbH, Germany; martin.kreutz@qiagen.com

12:15 **A Novel Simple and Inexpensive Assay for MicroRNAs Detection**
Irit Reichenstein, Zvi Bentwich and Yonat Shemer Avni
Ben-Gurion University of the Negev, Israel; Iritreic@bgu.ac.il

12:40 – 13:30 **Lunch in the student cafeteria**

Session **Single-cell qPCR**
Chair **B. Liss & A. Stahlberg**
Lecture hall **HS 15**

Session sponsored by:

13:30 **RT-qPCR of individual dopamine neurons form mouse brains and human post mortem brain sections.**
Birgit Liss
Molecular Neurophysiology, Institute for General Physiology, University of Ulm, Albert Einstein Allee 11, 89081 Ulm, Germany.; birgit.liss@uni-ulm.de

14:00 **Technical aspects of mRNA quantification in single cells using RT-qPCR**
Anders Ståhlberg
Gothenburg University, Sweden; anders.stahlberg@neuro.gu.se

14:25 **AmpliGrid and AmpliHyb, a new miniaturized, multiplex qPCR system for single cell analysis**
Gordana Cerovic[2], Marianna Alunni[1], Angelique le Bras[2], Régis Melizzi[2], Jean-Luc Grabias[2], Maxime Rattier[2], Martin Kantlehner[1], Petra Hartmann[1], Wolfgang Mann[1] and Claude Weisbuch[2]
[1]Olympus Life Science Research Europa, Germany; [2]Genewave, Palaiseau (France)
gordana.cerovic@genewave.com

14:50 **Post-characterization of cultured pituitary cells using single-cell real-time RT-PCR**
Kjetil Hodne, Trude M Haug, Olav Sand and Finn-Arne Weltzien
Dept of Molecular Biosciences, University of Oslo, Norway; kjetil.hodne@imbv.uio.no

15:15 **Molecular characterization of circulating tumor cells in large quantities of contaminating leukocytes by a multiplex real-time PCR**
ANIETA M SIEUWERTS[1], JACO KRAAN[2], JOAN BOLT-DE VRIES[1], PETRA VAN DER SPOEL[2], BIANCA MOSTERT[2], JOHN W MARTENS[1], JAN W GRATAMA[2], STEFAN SLEIJFER[2] and JOHN A FOEKENS[1]
[1]Department of Medical Oncology, Josephine Nefkens Institute and Cancer Genomics Centre, ERASMUS MC, Netherlands, The; [2]Department of Medical Oncology, Daniel den Hoed Cancer Center, ERASMUS MC, Netherlands, The; a.sieuwerts@erasmusmc.nl

15:40 **Visualization of Single mRNA Molecules**
Fay Wang[1], John Flanagan[1], Yunqing Ma[2], Steve Lai[2], Takuro Yaoi[2], Son Bui[2], Li-chong Wang[1], Jennifer Wong[1], Nan Su[1], Jessie Wu[2], Nina Nguyen[2], Aiguo Zhang[2], Steve Chen[1], Frank Witney[2], Quan Nguyen[2] and Yuling Luo[1]
[1]Advanced Cell Diagnostics, Inc., United States of America; [2]Affymetrix/Panomics, Inc., United States of America; yluo@acdbio.com

16:05 – 16:30 **Coffee break**

Session **Tutorials in qPCR BioStatistics & BioInformatics**
Chair **J. Vandesompele & A. Forootan**
Lecture hall **HS 15**

16:30 **Easy analysis of qPCR data with state of the art quantification models and comprehensive quality controls using qBasePlus.**
Jan Hellemans[1,2], Stefaan Derveaux[1] and Jo Vandesompele[1,2]
[1]Center for Medical Genetics, Ghent University, Belgium; [2]Biogazelle, Belgium; Jan.Hellemans@UGent.be

17:00 **RefGenes - a new tool to find suitable reference genes for selected experimental conditions**
Philip Zimmermann
ETH Zurich, Switzerland; phz@ethz.ch

17:30 **Data analysis for gene quantification and expression profiling using GenEx.**
Anders Bergkvist
MultiD Analyses AB, Sweden; anders.bergkvist@multid.se

18:00 **CAmpER - An open analysis framework for real-time PCR data using single sample amplification efficiency calculation.**
Jochen Blom[1], Lukas Jelonek[1], Jörn Kalinowski[2], Christian Rückert[2] and Alexander Goesmann[1]
[1]Bioinformatics Resource Facility, CeBitTec, Bielefeld University, Germany; [2]Institute for Genome Research, CeBiTec, Bielefeld Univeristy, Germany; jblom@cebitec.uni-bielefeld.de

Wednesday 11th March 2009

Session High-Throughout session
Chair J. Hellemans & K. Livak
Lecture hall HS 14

Session sponsored by:

8:30 **Moving from qPCR Assays to qPCR Arrays**
Kenneth James Livak
Fluidigm Corporation, United States of America; ken.livak@fluidigm.com

9:00 **Accurate and Objective Gene Copy Number Profiling using Real-Time PCR**
Jan Hellemans[2], Barbara D'haene[1], Frauke Coppieters[1], Steve Lefever[1], Filip Pattyn[1], Bart Leroy[1], Geert Mortier[1], Elfride De Baere[1] and Jo Vandesompele[2]
[1]Center for Medical Genetics, Ghent University Hospital, Belgium; [2]Ghent University, Belgium - Biogazelle, Belgium; Jan.Hellemans@UGent.be

9:25 **High-Throughput Analysis of Nucleic Acids Using the LightCycler® 1536 qPCR Platform**
Thomas Froehlich, Gregor Sagner, Gudrun Tellmann, Christian Weilke and Armin Tgetgel
Roche Diagnostics, Germany; thomas.froehlich@roche.com

9:50 **Measurement of Gene Expression by Massively Parallel Nanoliter real-time PCR**
Jim White
BioTrove, United States of America; jwhite1@biotrove.com

10:15 – 10:45 **Coffee break**

10:45 **QuantPrime – a flexible tool for reliable high-throughput primer design for quantitative PCR**
Samuel Arvidsson[1,2], Miroslaw Kwasniewski[1,2,3], Diego Mauricio Riano-Pachon[2] and Bernd Mueller-Roeber[1,2]
[1]Potsdam University, Germany; [2]Max-Planck Institute for Molecular Plant Physiology, Potsdam, Germany; [3]University of Silesia, Katowice, Poland; samuel.arvidsson@uni-potsdam.de

11:10 **Oligo Design Across the Mouse Genome**
Ben Sowers
Biosearch Technologies, Inc., United States of America; ben@biosearchtech.com

11:35 **A novel digital technology for non-enzymatic direct multiplexed measurement of gene expression**
Chaybani, Ramin, Gary K. Geiss[1], Roger Bumgarner[2], Brian Birditt[1], Timothy Dahl[1], Naeem Dowidar[1], Dwayne L. Dunaway[1], Perry Fell[1], Sean Ferree[1], Renee D. George[1], Tammy Grogan[1], Jeffrey J. James[1], Malini Maysuria[1], Jeffrey D. Mitton[1], Paola Oliveri[4], Jennifer L. Osborn[3], Tao Peng[2], Amber L. Ratcliffe[1], Philippa J. Webster[1], Eric H. Davidson[4] and Leroy Hood[5]
[1]NanoString Technologies Inc., Seattle, WA [2]Department of Microbiology, , University of Washington, Seattle WA; [3]Department of Bioengineering, University Washington, Seattle WA ; [4]Division of Biology, California Institute of Technology, Pasadena CA ; [5]The Institute of Systems Biology, Seattle WA; ramin@novoptim.com

12:00 **Sensitive and high throughput multiplexed immunoassays for biomarker discovery in biobanked samples using proximity ligation assays and qPCR.**
Simon Fredriksson
Olink Bioscience, Sweden; simon.fredriksson@olink.com

12:25 – 13:30 **Lunch in the student cafeteria**

Session qPCR BioStatistics & BioInformatics
Chair M. Kubista & A. Tichopad
Lecture hall HS 14

13:30 **Real-time PCR Expression Profiling - Concept of multiway profiling**
Mikael Kubista[1,2], Anders Ståhlberg[2,3], Jose Manuel Andrade[4], Björn Sjögreen[5,6], Amin Forootan[6] and Anders Bergkvist[6] [1]TATAA Biocenter, Sweden; [2]Institute of Biotechnology, Czech Academy of Sciences; Prague [3]Gothenburg University, Sweden; [4]University La Coruna, Spain; [5]MultiD Analyses AB, Sweden; [6]Lawrence Livermore Laboratory, USA; mikael.kubista@tataa.com

14:00 **Design and analysis of Q-RT-PCR assays for haematological malignancies using mixed effects models**
Martin Bøgsted[1,2], Charlotte Mandrup[1], Anders Petersen[1], Steffen Falgreen[1,2], Hans Erik Johnsen[1], Anne Bukh[1] and Karen Dybkær[1]
[1]Department of Haematology, Aarhus University Hospital, [2]Center for Cardiovascular Research, Aarhus University Hospital; Denmark; martin.boegsted@rn.dk

14:25 **Adequate experiment design as the first important step in obtaining valid biological inference with qPCR technique**
Ales Tichopad
TUM, Germany; ales@tichopad.de

14:50 **Modeling Real-Time PCR Efficiency and Fluorescent Signal intensity for accurate gene quantification using a single standard**
Kaminski Karine[1], Jahan Virginie[2], Lamoure Claire[2], Martineau Pierre[3] and Molina Franck[1]
[1]CNRS / Bio-Rad, France; [2]Bio-Rad, France; [3]IRCM INSERM, France; karine.kaminski@sysdiag.cnrs.fr

15:15 – 15:45 **Coffee break**

15:45 **The Delta-TF Method for Real-Time PCR Data Standardization**
Denis Rebrikov, Elena Goncharova, German Samatov, Pavel Semenov, Alexander Baluev and Dmitry Trofimov
DNA-Technology, Russian Federation; denis@dna-technology.ru

16:10 **Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data.**
Jan M Ruijter
Academic Medical Centre, Amsterdam, the Netherlands; j.m.ruijter@amc.uva.nl

16:35 **The calculation of real-time PCR ratios by means of Monte Carlo Simulation or high-order Taylor expansion.**
Andrej-Nikolai Spiess
University Hospital Hamburg-Eppendorf, Germany; a.spiess@uke.de

17:00 **rtprimerdb.org: public qPCR assay database with custom assay quality control and primer design pipeline**
Filip Pattyn, Steve Lefever, Frank Speleman and Jo Vandesompele
Ghent Univ. Hospital, Belgium; Filip.Pattyn@UGent.be

Closing of the Symposium
Lecture hall HS 14

17:30 **Closing of the Symposium**
Heinrich HD. Meyer & Michael W. Pfaffl

Wednesday 11th March 2009

Session: qPCR NOS Session (1)
Normalization & Optimization & Standardization
Chair: J. Vandesompele & S. Bustin
Lecture hall HS 15

Session sponsored by:

Lonza

8:30 **A new qPCR assay for the detection of Clostridium difficile**
Stephen A Bustin
Barts and the London School of Medicine, United Kingdom; s.a.bustin@qmul.ac.uk

9:00 **RealTime ready – Functionally Tested qPCR Assays for Gene Expression Analysis on the LightCycler® Platform**
Ralf P. Mauritz
Roche Diagnostics GmbH, Germany; ralf.mauritz@roche.com

9:25 **RDML: structured language and reporting guidelines for real-time PCR data**
Steve Lefever[1], Jan Hellemans[2], Filip Pattyn[1], Daniel Przybylski[3], Chris Taylor[4], René Geurts[5], Andreas Untergasser[5] and Jo Vandesompele[2]
[1]Center for Medical Genetics, Ghent University Hospital, Belgium; [2]Ghent University, Belgium - Biogazelle, Belgium; [3]Bio-Rad Laboratories, Inc. Hercules, California, USA; [4]European Bioinformatics Institute, Wellcome Trust Genome Campus, Hinxton, Cambridgeshire, CB10 1SD, UK; [5]Laboratory of Molecular Biology, Department of Plant Science, Wageningen University, The Netherlands; steve.lefever@ugent.be

9:50 **ZNA: new high-affinity synthetic oligonucleotides as powerful tools for PCR**
Nathalie Lenne[1], Valérie Moreau[1], Emilie Voirin[1], Régis Noir[2], Clément Paris[1], Mitsuharu Kotera[2], Jean-Paul Behr[2] and Patrick Erbacher[1]
[1]Polyplus-transfection, France; [2]Laboratoire de Chimie Génétique, Illkirch,France; nlenne@polyplus-transfection.com

10:15 – 10:45 **Coffee break**

10:45 **Importance of experimental design and sample QC for robust and meaningful QPCR results**
Steffen Mueller
Agilent, Germany; steffen.mueller@agilent.com

11:10 **Normalization of real-time RT-PCR data using an external RNA control**
Stian Ellefsen[1], Kåre-Olav Stensløkken[2,3], Guro Katrine Sandvik[3], Tom Arne Kristensen[3] and Göran Erik Nilsson[3]
[1]Lillehammer University College, Norway; [2]Ullevål University Hospital, Norway; [3]Department of Molecular Biosciences, University of Oslo, Norway; stian.ellefsen@hil.no

11:35 **Highly Accurate Quantitative Gene Expression Analysis without Use of pre-defined Normalizer Genes using Pattern Recognition Analysis**
Dan Shaffer, Volker Vogel and Don-Paul Kovarcik
Daniel J. Shaffer, VP and Founder, Bar Harbor Biotechnology, US, daniel.shaffer@barharborbio.com

12:00 **Optimisation and standardisation of sample preparation with the Bead-beating technology in q-PCR analysis.**
Romain VEROLLET and Esmeralda CARVALHO
Bertin Technologies, France; verollet@bertin.fr

12:25 – 13:30 **Lunch in the student cafeteria**

Session: qPCR NOS Session (2)
Chair: A. Nitsche & A. Stahlberg
Lecture hall HS 15

Session sponsored by:

Lonza

13:30 **Increasing QPCR throughput: simple steps to speed up results whilst minimising variance.**
Gerwyn Jones, Saima Nayab, Srujana Kapavarapu and Ian Kavanagh
Thermo Fisher Scientific, ABgene House, Blenheim Road, Epsom KT19 9AP United Kingdom; ian.kavanagh@thermofisher.com

13:55 **Significant difference or artefact of the method? - The impact of temperature performance of real-time thermocyclers on generated qPCR results**
Mary Span
CYCLERtest, Netherlands, The; marys@cyclertest.com

14:20 **The Next Generation in Hot Start PCR - CleanAmp Primers and dNTPs**
Natasha Paul
TriLink BioTechnologies, Inc., United States of America; npaul@trilinkbiotech.com

14:45 **Assessment of the reliability of nucleic acid extraction systems commonly used to get valid qPCR results**
Tom Øystein Jonassen[1], Mona Holberg-Petersen[1] and Einar S Berg[2]
[1]Ullevål University Hospital, Oslo, Norway; [2]Norwegian Institute of Public Health, Oslo, Norway; esbe@fhi.no

15:10 – 15:40 **Coffee break**

15:40 **Housekeeping genes validation in acute and chronic adjuvant arthritic rat for mRNA quantification by real time RT-PCR**
Muhammad Ayaz Alam Qureshi, Per Eriksson, Andrea Stark and Mahmood Ahmed
Karolinska Institute, Sweden; alam_ayaz@hotmail.com

16:05 **Gene Expression Analysis by Genome Controlled Reverse Transcription-PCR.**
Chas Andre[1], Jakob Stenman[2], Tuomas Tenkanen[1], Arto Orpana[2] and Susanna Lintula[2]
[1]Finnzymes OY, Finland; [2]Hospital for Children and Adolescents, Helsinki Finland; jakob.stenman@helsinki.fi

16:30 **QPCR Use in Biopharmaceuticals and Current Issues**
Chaminda Salgado
NDA Analytics, United Kingdom; chaminda.salgado@nda-analytics.com

Closing of the Symposium
Lecture hall HS 14

17:30 **Closing of the Symposium**
Heinrich HD. Meyer & Michael W. Pfaffl

Thursday 12th March 2009 & Friday 13th March 2009

qPCR Application Workshops

The workshops are aimed at giving participants a deep and objective understanding of real-time quantitative PCR, biostatistics, expression profiling, and its applications. The courses are intended for academic or industrial persons considering working with qPCR or scientists currently working with qPCR seeking a deeper understanding.

The qPCR courses cover all aspects in qPCR and are held during 2-days. Each course is approximately 50% hands-on and is limited to 20 participants (biostatistics 50 participants), resulting in very interactive teaching and everybody given the opportunity to try the instrumentation. After the course participants will be able to plan and perform qPCR experiments themselves, as well as interpret and analyze data. Detailed course material and full catering (lunch, coffee, soft drinks and snacks) are included in the course fee.

Workshops starts on **Thursday and Friday at 9 am until 5 pm**. All four workshops are hosted by the TATAA Biocenter Sweden, TATAA Biocenter Germany, and bioEPS GmbH (www.tataa.com , TATAA.gene-quantification.info , www.bioeps.com). The TATAA qPCR workshop laboratories and seminar rooms are close to the central lecture hall.

Workshop topics:

- Basic Module qPCR Application Workshop (2-days) — Practical room – **P2**
- Sample Preparation Workshop (2-days) — Practical room – **P3**
- High Resolution Melt Workshop (1-day) & Immuno-qPCR (1-day) — Practical room – **P4**
- qPCR Biostatistics & Expression Profiling Workshop (2-days) — Computer seminar room – **PU**

Basic Module qPCR Application Workshop (2-days) — Practical room – P2

The introductory course consists of a theoretical part and a practical part where participants get to do QPCR experiments by themselves under experienced supervision. The course contains:

Day 1:
- Basic PCR theory
- The theory of real-time PCR
- Applications and possibilities of QPCR. Comparison of QPCR with regular PCR.
- Review of currently available detection technologies (SYBR Green I, TaqMan, Molecular Beacons...etc)
- Different instrument plattforms and their typical uses
- Primer Design
- The problem of primer-dimer formation and how to minimize them
- Probe Design
- Experimental design and optimization
- Basic data handling and analysis

Day 2:
This course covers aspects in sample preparation and reverse transcriptions.
- Principles of RT
- Priming methods for RT
- What enzymes are preferential for different applications
- Sample Preparation (Extraction of RNA and DNA)
- Introduction to statsitics and statistical analysis of data

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qPCR Biostatistics & Expression Profiling Workshop (2-days) — Computer seminar room – PU

This course explains statistics applicable to qPCR and teaches how to use statistics to interpret real-time PCR gene expression data, and classify samples based on real-time PCR expression profiling. Course is based on seminars and computer-based demonstrations. Please bring your own Laptop to the course!

Day 1 - Statistical analysis of real-time PCR data
Lectures cover the principles of statistics, including Gaussian statistics, the central limit theorem, p values and statistical hypothesis testing, z-scores, rank-based methods (non-Gaussian), comparison of two groups (paired and unpaired t-test), Mann Whitney test, Wilcoxon rank sum test, Fisher's exact test. Outlier detection (Dixon's test, Grubb's test, Cochran's test), ANOVA and classical calibration (least square fit, correlation coefficient, Hottelings' area). During computer based workshop participants will learn how to analyze typical real-time PCR data sets. Examples include identification of outliers, and how to compare means and variances of paired and unpaired studies.

Day 2 - Gene expression profiling with real-time PCR
Lectures cover methods to classify samples and genes. The methods presented include Principal Component Analysis, Potential Curves, Hierachical Clustering, Self-Organizing Maps, and Trilinear Decomposition. During computer based workshops participants will classify metabolic genes in yeast, developmental stages in Xenopus laevis, Breast cancer data, and developing stem cells.

Sample Preparation Workshop (2-days) Practical room – P3

One of the most important requirements to get good results from qPCR experiment is to have a template of good quality. In most cases this means having an efficient sample preparation. This course module is focused on extraction of RNA and DNA to be used as template in qPCR and reverse transcription reactions. The course covers:

- Overview of nucleic acid extraction methods
- How to properly determine the concentration of purified nucleic acids
- Extraction from limited amount of material and single cells
- Storage biological samples and purified nucleic acids
- Quality control of purified material
- Integrity of purified RNA
- How to test for the presence of inhibitors

High Resolution Melt Workshop (1-day) & Immuno-qPCR (1-day) Practical room – P4

High-Resolution-Melting Workshop (on day 1)

This is an introductory course in HRM, where a high resolved melting curve is used to analyze very small differences in melting temperature of PCR products, differences that can be due to a single base substitution. The course includes seminars as well as hands on training where the participants get to perform experiments. The course covers:

- Introduction to HRM
- Assay design
- SNP – analysis
- Genescanning
- Methylation analysis
- Review of available HRM instruments
- Examples of other applications

Immuno-qPCR Workshop (on day 2)

This course shows how real-time PCR can be used to quantify proteins. The course consists of a theoretical part which explains what immuno-qPCR is and how it can be set up and used. The course also includes a practical part where the course participants will run an immuno-qPCR experiment to quantify a protein. The course covers:

- Introduction to immunoassays.
- How to set up an immuno-qPCR assay.
- How to optimize an immuno-qPCR.
- How to analyse immuno-qPCR data.
- Troubleshooting.
- Examples of immuno-qPCR applications.
- Practical experiment quantifying a protein.

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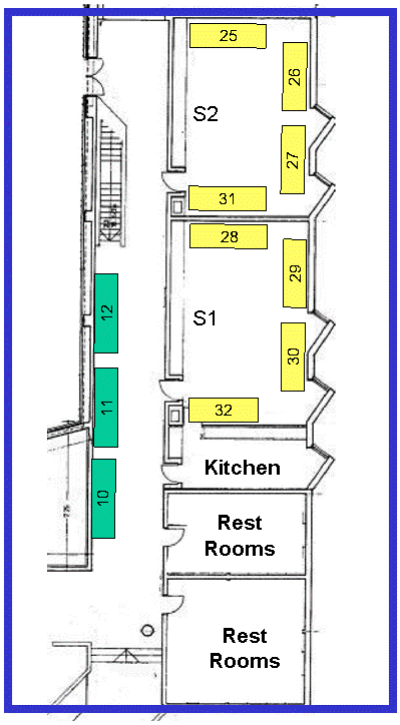
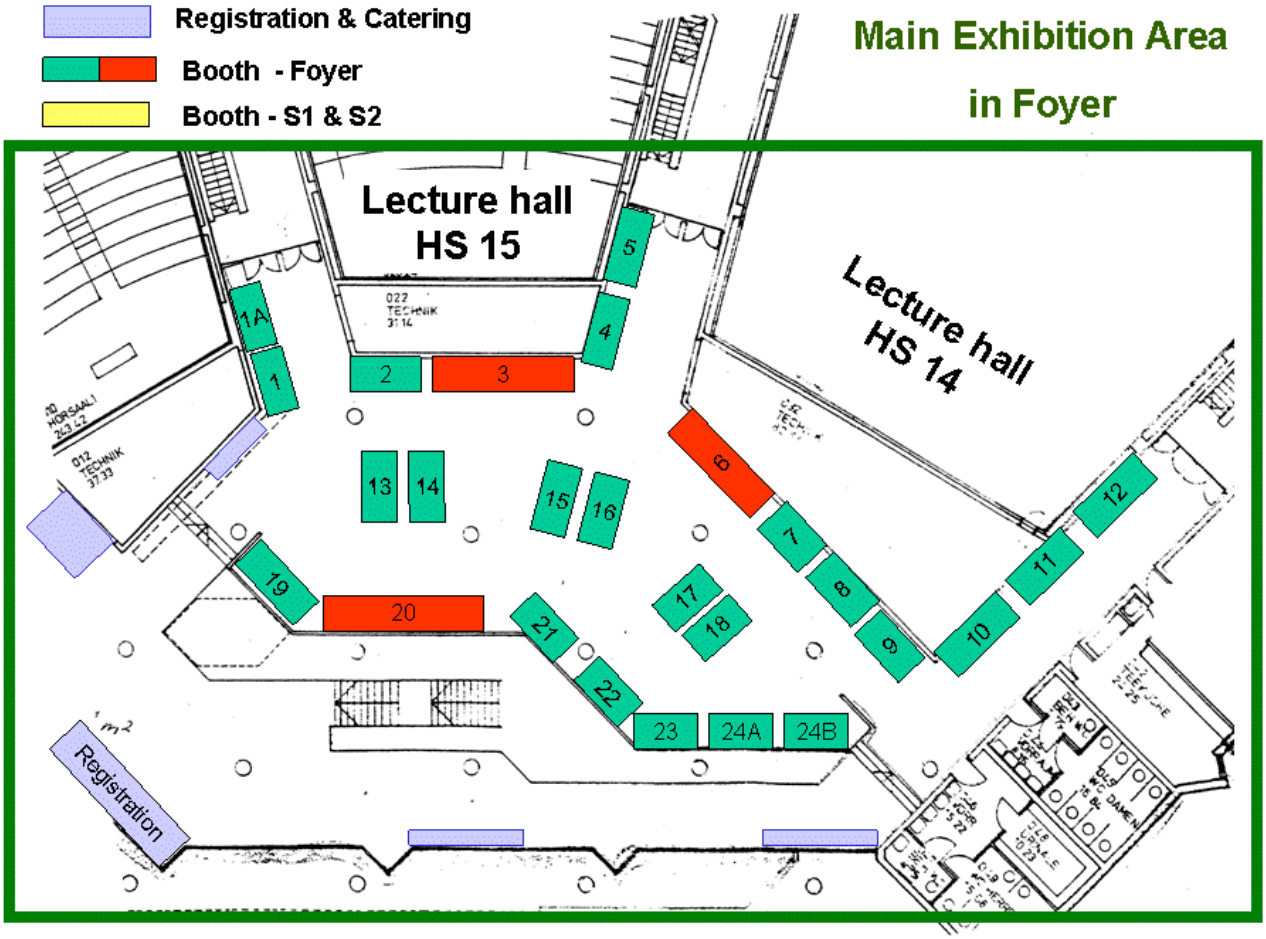
qPCR Workshop Sponsors:

Industrial Exhibition

More than **35 companies** participate at the industrial exhibition held during the qPCR Symposium March 9th – 11th in the foyer of the central lecture hall complex (green frame) and in two side rooms S1 and S2 (blue frame).

Booth	Company	Booth	Company
1	Eppendorf	17	Metabion
1A	Finnzymes / Biozym	18	Biomol
2	Biosearch Technologies	19	TIB Molbiol
3	Bio-Rad	20	Fluidigm
4	Biotec Pharmacon	21	4titude
5	Lonza	22	Applied Biosystems
6	Roche Applied Science	23	Invitrogen
7	Agilent Technologies	24A	Beckman Coulter
8	Advalytix / Olymus	24B	Primer Design
9	AJ Roboscreen / AJ eBiochip	25	Nanostring
10	Integrated DNA Technologies	26	Biogazelle
11	Exiqon	27	Biolegio
12	Trilink Biotechnologies	28	Alphametrix / Biotrove
13	Eurofins MWG Operon	29	Helixis
14	Eurogentec	30	MP Biomedicals
15	Thermo Fisher Scientific	31	LabOnNet
16	Qiagen	32	MultiD





Abstracts - Oral presentations

Monday 9th March 2009

HOT TOPIC - KEYNOTE LECTURE

MIQE- guidelines for publication of qPCR data

Stephen A Bustin[1], Vladimir Benes[2], Jeremy A Garson[3], Jan Hellemans[4], Jim Huggett[3], Mikael Kubista[5], Reinhold Mueller[6], Tania Nolan[7], Michael W Pfaffl[8], Gregory L Shipley[9], Jo Vandesompele[4] and Carl T Wittwer[10]
[1]Barts and the London School of Medicine, UK; [2]Genomics Core Facility, EMBL Heidelberg, Germany; [3]University College London, UK and UCL Hospitals NHS Foundation Trust, UK; [4]Ghent University Hospital, Belgium; [5]Institute of Biotechnology AS CR, Czech Republic and TATAA Biocenter, Sweden; [6]Sequenom, USA; [7]Sigma-Aldrich, UK; [8]Technical University Munich, Germany; [9]University of Texas Health Science Centre, USA; [10]University of Utah USA and ARUP Institute for Clinical and Experimental Pathology, USA; s.a.bustin@qmul.ac.uk

MIQE is a set of guidelines that describe the minimum information necessary for evaluation of quantitative real-time polymerase chain reaction experiments. Included is a checklist to accompany the initial submission of a manuscript to the publisher. By providing all relevant experimental conditions and assay characteristics, reviewers can assess the validity of the protocols used. Full disclosure of all reagents, sequences, and analysis methods is necessary to enable other investigators to reproduce results. MIQE details should be published either in abbreviated form or as an online supplement. Following these guidelines will encourage better experimental practice, allowing more reliable and unequivocal interpretation of quantitative PCR results.

Session Diagnostic & Molecular Markers (1)
Chair J. Huggett & MW. Pfaffl
Lecture hall HS 14

Session sponsored by:

A Multi-Assay Approach to the Study of Cellular Toxicity

Gregory L Shipley
UTHSC-Houston, United States of America; gregory.l.shipley@uth.tmc.edu

The screening of cells against a large number of chemical compounds with diverse biological effects can result in false positive 'hits' due to cellular toxicity rather than specific effects on the biological target of interest. The problem is even more acute when the assay end-point is cell death. In an effort to determine the most expeditious method of finding compounds that are toxic to a particular cell type, we have embarked on a multi-assay approach. Cellular assays include following cells prior and during treatment with the Roche xCelligence real-time cell analysis instrument and post-treatment high content cell imaging assays. At the molecular level, we are using end-point viability assays and a battery of real-time RT-qPCR assays utilizing whole cell lysates.

Circulating nucleic acids in melanoma diagnosis

Pamela Pinzani[1], Francesca Salvianti[1], Roberta Cascella[1], Vincenzo De Giorgi[2], Daniela Massi[3], Mario Pazzagli[1] and Claudio Orlando[1]
[1]Department of Clinical Physiopathology, University of Florence, Florence, Italy; [2]Department of Dermatological Sciences, University of Florence, Florence, Italy; [3]Department of Human Pathology and Oncology, University of Florence, Florence, Italy; p.pinzani@dfc.unifi.it

Circulating nucleic acids represent important biomarkers used as diagnostic and prognostic tools in oncology. Nucleic acids can be associated to circulating tumor cells (CTC) or free in blood plasma. The most accepted technique for the detection of cell-associated circulating nucleic acids is real time RT-PCR for a specific mRNA. As regard to the evaluation of circulating nucleic acid (CNA), it is possible to evaluate either the total DNA plasma concentration or alternatively the concentration of specific DNA sequences characterized by tumor-specific genetic or epigenetic changes. During the last decade we have witnessed significant progress in understanding the genetic and epigenetic changes involved in the development and progression of cutaneous melanoma, including the identification of high frequency occurrence of somatic mutations in the BRAF gene. The more frequent is the V600E, which leads to increased proliferation, invasion and survival of melanoma cells via the activation of MAP kinase pathway. Furthermore, the methylation of RASSF1A promoter is one of the epigenetic changes identified in melanoma. By real time PCR, we used the APP gene for the measurement of the total amount of circulating DNA, while BRAFV600E mutation and RASSF1A promoter methylation have been employed as biomarkers of tumor-related CNA in melanoma. By real time RT-PCR, we measured tyrosinase mRNA expression (a key enzyme in the synthesis of melanin) in the whole blood as a marker of presence of CTC. The aim of our research was to assess the specificity and sensitivity of these four biomarkers that were tested in the blood of 62 patients affected by melanoma at different stages, 7 patients with non-melanocytic tumors, 7 subjects with benign melanocytic lesions (nevi) and 18 healthy control subjects. The analyzed parameters showed a significant increase in patients affected by melanoma compared to controls, but none of them showed a statistical difference between control subjects and patients with nevi or non-melanocytic tumors. Clinical sensitivity and specificity were assessed for each parameter, by comparing their ROC curves. Fixing the specificity value at 100%, the four indicators achieved a diagnostic sensitivity of 65% for APP, 49% for BRAF, 45% for RASSF1A and 25% for the tyrosinase. 34% of patients were positive for only one of the above mentioned markers, 35% for 2 of them, 25% for three and only 2% of cases for all the four parameters. Only two patients were negative for all the genetic or epigenetic modifications, with a resulting overall diagnostic sensitivity of 96%. In conclusion, our data suggest that only the simultaneous determination of several circulating biomarkers significantly increases the diagnostic sensitivity in cutaneous melanoma. Consequently, we propose a diagnostic approach based on the sequential determination of APP, BRAF, RASSF1A and, finally, tyrosinase mRNA.

Prognostic multigene expression classification of cancer patients: a route for success

Joëlle Vermeulen[1], Katleen De Preter[1], Filip Pattyn[1], Liesbeth Vercruysse[1], Nurten Yigit[1], Jan Hellemans[2], Frank Speleman[1] and Jo Vandesompele[2]
[1]Center for Medical Genetics, Ghent University Hospital, Belgium; [2]Ghent University, Belgium - Biogazelle, Belgium; joke.vandesompele@ugent.be

More accurate assessment of prognosis is of importance to improve the choice of risk-related therapy in cancer patients. It has been shown that gene expression profiling is able to achieve this goal. Here, we outline a strategy for real-time PCR based gene expression profiling of 600 neuroblastoma patients using 60 prognostic markers identified upon meta-analysis of published gene expression studies. Key components of the strategy are the testing of many more patients than genes, rigorous RNA quality control, thorough evaluation of qPCR gene expression assays, use of absolute standards for cross-laboratory comparison, and application of a pre-amplification procedure enabling the profiling using only 20 ng of total RNA as starting material. Following the outlined strategy, we established a robust and accurate prognostic multigene expression predictor, suitable for routine lab tests and ready to be evaluated in prospective studies.

Session **Diagnostic & Molecular Markers**
Session part 2
Chair **S. Bustin & G. Shipley**
Lecture hall **HS 14**

Session sponsored by:

A Novel Multiplex, Quantitative Gene Expression Approach for Cancer Biomarker Research
Jim Thorn
Beckman Coulter UK, UK; JTHORN@beckman.com

Quantitative gene expression analysis is playing an increasingly important role in cancer research. Currently available techniques either utilize microarray to detect the expression of a high number of genes per reaction at high cost, or utilize real-time PCR† to detect the expression of a few genes at low throughput. A multiplex approach to analyze a set of multiple genes from a given biological pathway in a single reaction using a limited amount of total tissue RNA is of great interest to cancer biologists. To address this need, the GenomeLab™ GeXP Genetic Analysis System was developed utilizing eXpress Profiling, a patented, highly multiplexed PCR approach to quickly and efficiently look at the expression of 20-30 multiplexed gene sets with greater sensitivity. The throughput is scalable from 80 to over 4000 gene expression results per day. Here we present a study of two cancer panels: a set of 29 genes directly or indirectly related to breast cancer formation; and the second set of 20 genes for metastasis progression. These two multiplex gene expression marker panels were developed not only for breast cancer detection, but also to differentiate cancerous stages. Our study demonstrated that this novel approach can quantitatively detect the expression of both cancer marker panels in a single reaction using as little as 25 ng of total RNA isolated from human breast cancer tissues and other types of cancerous tissues. The GenomeLab GeXP System has proven to be a cost-effective way of performing multiplex gene expression analysis with scalable throughput capacity, high assay robustness, and excellent data quality.

The use of nucleic acid amplification tests for research and diagnosis in the developing world.
Jim Francis Huggett[1], Clare Green[1], Michael Hoelscher[2] and Alimuddin Zumla[1]
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Nucleic acid amplification tests (NAATs) have impacted less on research and clinical diagnosis in the developing world than they have in the developed world. This is not surprising when the costs and infrastructure required to perform NAATs are considered. However NAATs are increasingly being used both for research and diagnosis in countries like Zambia and Tanzania. NAATs play an important role in capacity development of developing world laboratories, as methods like PCR (both legacy and real time) are central to many aspects of clinical, agricultural and biological research. This is made possible through the support and development of the necessary laboratory infrastructure. This has enabled world-leading research to be performed on essential research- from rapid diagnosis of infectious diseases to RNA gene expression measurements to monitor immune responses- by the countries most affected by the diseases or other questions under investigation. Concomitantly the challenge presented by the need for tests that are more appropriate for a resource poor setting has lead to a range of innovations. These include newer methodologies for nucleic acid detection, which can be tailored to perform in the field without the need for training in molecular biology, to mobile laboratories that take advanced molecular tests to the rural populations. With the emergence of serious infectious threats, like extremely drug resistant tuberculosis, in which antibiotics are effectively rendered useless, NAATs represent a valuable infection control tool. As NAATs become both more simple and cheaper their impact is likely to play an increasingly crucial role in developing world research and diagnosis.

Effective placement of LNA into Q-PCR Probes
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Locked Nucleic Acid (LNA) chemistry, introduced into a Q-PCR probe, increases thermal duplex stability and improves specificity of probe hybridization to its target sequence. It provides more accurate gene quantitation and allelic discrimination, and more flexible probe designs for difficult target sequences. There are also potential benefits of LNA in multiplexing. LNA placement within the probe is critical to using the chemistry effectively; understanding the thermodynamic properties of LNA, through empirical laboratory work, is the key to effective placement. Celadon Laboratories and Jason Kahn, PhD of the University of Maryland's Department of Chemistry and Biochemistry has spent more than five years, under government research contracts, in generating comprehensive melt curve data for LNA -incorporated oligonucleotides. These LNA hybridization rules can be incorporated into our assay-design software for best prediction of effective LNA placement. In this presentation, Raymond Peterson, PhD, founder and Chief Scientific Officer of Celadon, will discuss the process of melt-curve generation and the resulting placement rules.

Expression signatures in IBD classification: A new approach
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Background & Aims: Inflammatory bowel disease (IBD) and inflammatory bowel syndrome (IBS) are heterogeneous disorders of the gastrointestinal tract and can profoundly affect the quality of life. Because many of the symptoms of IBD are similar to those of IBS, the former may be misdiagnosed. Additionally, the two major forms of IBD, ulcerative colitis (UC) and Crohn's disease (CD) show characteristic, but non-specific, pathological features that may overlap and result in a long delay of a final diagnosis or a diagnosis of "unspecified IBD" (IBDU) or "indeterminate colitis" (IC). The aim of our studies was to identify and confirm the usability of specific expression markers as a means to clinically distinguish IBS from IBD and to discriminate UC from CD. *Methods:* We used a PCR-based cDNA subtraction method to identify IBD specific genes from endoscopic biopsies. In quantitative PCR experiments, the differential expression of the identified genes were analysed and the clinical utility of these marker genes were evaluated in over 300 patients using a special constructed algorithm. *Results:* Seven marker genes were identified as differentially expressed in IBS and IBD as well as in UC and CD: three genes involved in cross membrane transport (SLC6A14, SLC26A2 and SPAP), three genes with a described function in tissue repair/remodelling and inflammation (Reg IV, Vanin-1 and MMP-7) and one gene playing a direct role in inflammation (GRO-α). Each of these markers has a high discriminating potential but in combination they reflect distinct expression signatures that are remarkably similar for patients of the same disease. Using the differential expression profiles of these genes we developed a rapid, sensitive and reproducible method that combines fluorescence-based real-time PCR and a special constructed algorithm for quantification and analysis of the expression signatures derived from the seven genes. In three independent studies we could confirm the diagnosis of the physicians in 85% to 98% of all cases. The specificity of having IBD was ranging from 94% to 95% with a sensitivity of 85% to 95% and the specificity of having UC from 86% to 99% with a sensitivity of 88% to 96%. *Conclusions:* The studies demonstrated the utility of applying expression profiling to identify expression markers from biopsies of patients with IBD and/or IBS whose collective expression signatures could form the basis for a quick and reliable diagnosis and ultimately improve disease management.

Intragraft expression profiles by quantitative PCR in kidney transplant patients reflect variability in the response to anti-rejection treatment with corticosteroids
Niels Rekers[1], Ingeborg Bajema[2], Kim Zuidwijk[3], Marko Mallat[3], Natascha Goemaere[4], Marian van Groningen[2], Cees van Kooten[3], Hans de Fijter[3], Frans Claas[1] and Michael Eikmans[1]
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Steroid resistant rejections are a major risk factor for adverse long-term graft outcome in kidney transplantation. We are aiming to predict response to anti-rejection treatment with steroids on the basis of molecular markers in the graft tissue.
All renal patients transplanted in our center between 1995 and 2005, who had a first acute rejection episode, and who received steroid treatment after the biopsy, were included. Steroid resistance was defined as requirement for ATG treatment within 2 weeks after steroid administration. For 101 patients frozen biopsy tissue was available for RNA extraction and cDNA synthesis. Messenger RNA expression of 47 markers was assessed by quantitative PCR (qPCR). The included markers together reflect the whole spectrum of immune cells (CTLs, Th cells, Tregs, NK cells, macrophages, granulocytes, B cells, plasma cells, mast cells) and several cytokines. We chose for the qPCR approach, since many of the low-expression immune transcripts cannot be detected by conventional microarray platforms. Expression of the markers was normalized for the average signal of three different reference genes (18S rRNA, GAPDH, β-actin). Primers for all markers tested fulfilled the following criteria: 1) PCR efficiency was 90-110%, 2) melt curve analysis displayed a single sharp peek with low background signals, 3) genomic DNA contaminants in the cDNA were not amplified. Steady-state expression levels were logarithmically transformed. Patients were hierarchically clustered based upon gene expression profiles using Spotfire (Tibco Software Inc.).
The patient cohort fell apart into four clusters with distinct expression profiles. Cluster 1 (n=9) displayed a transcriptionally quiescent profile. In this cluster 89% of the rejections were steroid sensitive. Cluster 2 (n=27) resembled cluster 1, apart from a slight upregulation of some markers. In this cluster 70% of the rejections were steroid sensitive. In comparison to the first two clusters, cluster 3 (n=44) and cluster 4 (n=21) contained significantly more steroid resistant rejections, i.e., 51% (P=0.005). Cluster 3 and 4 had in common an extensive upregulation of markers reflecting T and B cell activation (CD69, CD127, HLA-DR, CD25, Lag3, CD86, ICOS, IL-10, IL-16, IL-21) and plasma cells (IgG, IgM, Blimp-1). The distribution of rejection severity in cluster 1-2 (borderline changes: 44%, Banff I: 25%, Banff II/III: 31%) differed from that in cluster 3-4 (borderline changes: 22%, Banff I: 44%, Banff II/III: 34%) (P=0.045).
Differences in intragraft expression profiles are observed in patients with a rejecting kidney transplant, and partly reflect variability in the response to anti-rejection treatment with corticosteroids. The qPCR method may be an informative means to complement clinical and pathologic evaluation for diagnostic purposes. The current findings suggest an association of high transcript levels of active T cells, B cells, and plasma cells with steroid resistance.

Session **Nobel Prize Laureate Lecture**
Chair **R. Cook & MW. Pfaffl**
Lecture hall **HS 14** **16:30 – 18:00**

Nobel Prize Laureate Kary Mullis

25th Anniversary of PCR

Session sponsored by:

The Conception, Early proof, and Cool Reception of the Invention of PCR: the Parallels between that and Almost Every Significant Invention to Date With Specific Reference to the Altermune Method in 2008
Kary Mullis. Ph.D., Nobel Prize Laureate
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Novelty, very distinct from what would reasonably be expected from people paid to be innovators, scientists and imaginative engineers, is not highly sought after by that group. Incremental, inconsequential innovations attract the unyielding and immediate attention of this, the scientific establishment. Editors, funders and managers are happy to support trivial contributions to what is mistakenly seen as the inevitable, glacial advance of science. Very few scientists, although formally paying heed to the notion that ultimate scientific progress consists in leaps and bounds and drastic paradigm shifts, are willing to recognize change soon after or while it is happening. As soon as it reaches a critical mass, cannot practically be denied, they are suddenly all on board.
In this discussion, attempting to take all this in stride, I will try to describe how this feels from the inside for someone who is strictly an innovator without an iota of political or business savy. It can't be all that much fun, but it is.

Session **High Resolution Melting & Genotyping**
Chair **J. Hellemans & A. Stahlberg**
Lecture hall **HS 15**

Applications of HRM curve analysis: strengths and pitfalls.
Kim De Leeneer, Ilse Coene, Bruce Poppe, Anne De Paepe and Kathleen Claes
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High-resolution melting curve analysis (HRMCA) is an emerging technology for detection of nucleic acid sequence variations. High-resolution melting curve analysis of nucleic acids depends on the ability to record and evaluate fluorescence intensities as a function of the melting temperature of PCR products. Developments in instrumentation and saturating intercalating dyes have made accurate high-resolution melting analysis possible and created opportunities to use this technology in mutation scanning. . We have recently optimized and validated HRMCA for mutation screening of the BRCA1/2 genes. Since distinct genetic variants can generate similar melting curves, every aberrant fragment needs to be sequenced to distinguish polymorphic variants from true pathogenic mutations. Due to the high polymorphic character of BRCA1 and BRCA2, the sequencing involved, after HRMCA as pre-screening method, still remains labour-intensive. To further reduce the sequencing burden, we optimised unlabeled probe genotyping protocols for the most prevalent SNP's in our population. The optimisation of these two HRMCA applications has made screening of large polymorphic genes fast, reliable and very cost-effective.

Using Melt Curve Analyses for Experimental Inquiry
Madeline O'Donoghue, Junko Stephens, Nathalie Koch, Jonathan Wang, Gordon Janaway, Laurel Nelson
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Since the release of the first Real-Time PCR system in 1996, the ABI Prism 7700, DNA melt curves have been a useful tool for evaluating the quality of a real-time reaction. More recently, the melt curve technique has expanded into two exciting areas: High Resolution Melting and Protein Thermal Shift Assays. Standard DNA melting curve analysis is a post-PCR method where a melting curve is generated by slowly denaturing (melting) amplicons through a range of temperatures in the presence of a dsDNA binding dye. Fluorescence decreases as temperature increases, a result of the dye being released when the two DNA strands denature. The inflection point of the curve, otherwise known as the T_m of the amplified material, is used to characterize the PCR products and is a function of GC-content (T_m is higher in GC-rich PCR products), length, and sequence content. Although this approach does provide some measure of amplicon identity, sensitivity is limited and the standard Real-Time PCR melt curve is most commonly used to detect primer-dimers or other non-specific by-products in PCR reactions. High Resolution Melting (HRM) is a relatively new, post-PCR analysis method used for identifying genetic variation in nucleic acid sequences. HRM is much like the standard Real Time melt approach in that the unique characteristics of each novel amplicon will contribute to the melt profile, however there is an increase in the resolution of data points in an HRM curve allowing for discrimination of DNA sequence differences of a single base pair between two

amplicons. HRM profiles can provide valuable information for mutation screening, genotyping, and other investigative applications. The Protein Thermal Shift Assay (TSA) is another different approach, where the dissociation profile of a Real-Time PCR Instrument is used as a rapid and sensitive screening tool in the absence of PCR. TSA measures protein denaturation using non-specific dyes, such as SYPRO® Orange, that will fluoresce in the presence of a denaturing protein. This rapid and sensitive tool is used for monitoring protein-ligand interactions or in the identification of optimal conditions or conformations/sequences that favour protein stability. The inflection points of individual curves in a TSA are used to compare different test conditions, and the extent of the observed temperature shift is considered to be representative of the stability of the protein in certain solution conditions. These three different melt curve approaches have all been used successfully on the full range of Applied Biosystems Real-Time PCR Instruments, allowing for increased flexibility to meet a variety of research needs.

Probe Based Detection of Genetic Variations - Screening and in-vitro Diagnostics

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Real-Time-PCR is employed for detection (quantification) and identification (typing). High resolution melting (HRM) and probe based methods are compared for their potential and limitations. Allele specific primers / Scorpions primers as well as TaqMan probes may report false results in case of new variations, whereas melting curve based methods will yield a correct result. Data for MTHR and lactose intolerance suggest that HRM is not capable to differentiate multiple variations, while probe based melting data report specific temperatures. For applications where more than 5-10 variants have to be identified the combination of qPCR and a low-density Chipron array provides both quantity and type information. Last not least it is shown for k-ras, BRAF in cancer and Glivec and Tamiflu resistent mutation search that hybridization probes and arrays can combined with a competitor to detect minimal amounts of said variants.

Releasing the potential of High Resolution Melting analysis

Rob Powell
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HRM holds great promise as the genotyping method of choice for the future. HRM will also impact many other related fields such as new SNP identification, DNA fingerprinting, methylation analysis and acquired mutation ratios. In order to fulfill this potential researcher must have access to high quality and complete solutions that can be used in a wide range of formats on multiple hardware platforms. PrimerDesign has been working towards these aims and is pleased to present research notes on a new mastermix for HRM. The formulation has been optimized for enhanced product yield to give maximum sensitivity in HRM analysis. This mastermix has been combined with a new cyanine based dye to give a completely new generation of HRM mastermix. The quality of data is superior to results obtained with SYTO9 and other widely used HRM dyes on the rotorgene 6000. We have optimized a probe based SNP detection system for melting analysis similar to the Lunar probes. The Tm differences seen between genotypes with this system are much larger than those obtained by melting the amplicon alone. We have shown that this format can be used on machines that have a standard melting curve function and do not require high resolution of melting to obtain accurate data. Overall our research will open a new era of genotyping by melting analysis on a wide range of platforms.

Simultaneous Determination of SNP Genotype and Allelic Copy Number of DME Gene CYP2D6

Adam Broomer, Toni Ceccardi, Kelly Li, Yu Wang, Chunlin Xiao and Caifu Chen
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The accuracy of genotyping single nucleotide polymorphisms (SNP) is crucial toward successfully determining disease associations and drug metabolism phenotypes. However, the presence of copy number variation (CNV), which is estimated to account for 12% of the human genome, may impair such studies. For example, samples containing a CNV region inclusive of the SNP locus may provide genotypes of A and B (i.e. deletion) or AAA, AAB, ABB, and BBB (i.e. duplication). The genotypes from such samples may be called incorrectly in the context of the usual genotypes of AA, BB, and AB from samples containing only two copies of the SNP locus. Here, we report a method for simultaneously determining both allelic identity and copy number from a SNP. The method employs a 2-plex TaqMan® PCR reaction comprising of an allele-specific assay and an endogenous control assay for each allele to be interrogated. A control sample for each allele then provides the allelic copy number for the unknown samples via the 2-ΔΔCT calculation method. The method is demonstrated on the CYP2D6*2 and CYP2D6*45A of the gene CYP2D6, which encodes a cytochrome P450 monooxygenase responsible for the metabolism of approximately 20% of prescribed drugs. The results are discussed in the context of the genotypes and gene copy numbers as determined by the TaqMan® DME Genotyping assays and TaqMan® Copy Number assays, respectively. Simultaneous determination of DNA copy number and its genotype for DME genes will be a powerful tool for Pharmacogenomics and personalized medicine.

Haematopoietic Chimerism Analysis after Allogeneic Stem Cell Transplantation

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After a haematopoietic stem cell transplant it is valuable to assess engraftment by monitoring the proportions of donor and recipient cells in the blood. Determining the mix of cells, or 'chimerism', provides useful information for the scheduling of specific therapeutic interventions, such as the withdrawal of immunosuppressive drugs or administration of donor lymphocyte infusions. The aim of this study was to extend whole blood chimerism analysis to a lineage-specific test. The additional information provided could help with understanding the dynamics of the engraftment process and facilitate more effective treatment in the post-transplant period.
In our routine monitoring, whole blood chimerism is measured by tracking a simplified genetic fingerprint of polymorphic short tandem repeats (STR's) that uniquely define the donor and recipient. PCR reactions using three STR markers are set up using a commercially available forensics kit. The differently sized fluorescent PCR products are detected and analysed on a capillary system genetic analyser. An assessment of the proportions of donor and recipient chimerism is calculated from the analyser output using a simple algorithm measuring the peak height and area. In developing a lineage-specific analysis, leukocyte sub-types were isolated by cell separation using AutoMACS® immuno-magnetic separation technology. DNA was isolated from the purified fractions and levels of chimerism analysed as for whole blood. Of eight patients studied, two patients displayed full donor chimerism in both the whole blood and lineage-specific analysis. Three patients with whole blood donor chimerism of less than 100% showed more pronounced fluctuations in lineage-specific donor chimerism. One patient, who consistently showed whole blood donor chimerism of 90-95%, displayed a similar level of donor chimerism in the myeloid fraction, but a more significant decrease to 55% in the T-cell (CD3) fraction. This patient received a stem cell transplant after refractory chronic myeloid leukaemia (CML) and so this result could give useful information as to whether the chimeric state was due to a recurrence of the original myeloid (CD33) clone. Reduced T-cell chimerism is strongly associated with graft failure and the low donor CD3 value could also provide evidence as to the success of the engraftment.
This preliminary study has confirmed that lineage-specific chimerism analysis represents a valuable adjunct to whole blood studies. Due to its increased sensitivity it can be used to reveal mixed chimerism in specific leukocyte populations that are masked in the whole blood analysis. Information about the relative proportions of donor and recipient T-cells is important in understanding the dynamics of engraftment and predicting graft vs. leukaemia and graft vs. host effects. Consequently, T-cell chimerism represents a particularly valuable part of the cell-specific analysis.

Tuesday 10th March 2009

Session Diagnostic & Molecular Markers (3)
Chair U. Reischl & H. Nitschko
Lecture hall HS 14

Session sponsored by:

CURRENT APPLICATIONS OF REAL-TIME PCR TECHNOLOGY IN DIAGNOSTIC BACTERIOLOGY

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For more than a century, pathogenic microbial detection and identification have relied on the ability to cultivate organisms in the diagnostic laboratory. Since the event of PCR about twenty years ago some of the limitations associated with these traditional methods were complemented by culture-independent molecular methods for pathogen detection and characterization. And, more recently, the clear advantages of real-time PCR technology (like speed, closed assay formats and melting curve analysis of hybridization probes) have been made accessible even for routine laboratories. Due to the widespread application of real-time PCR and the development of various assay formats, a huge spectrum of diagnostic in-house protocols or commercial kits are now available to detect almost any bacterial or fungal organsim as well as some of the most relevant pathogenicity factors - and with the ongoing process of identifying valuable target genes or characteristic mutations there is enough space for additional diagnostic protocols and assay developments. The versatility of this approach is exemplified by applications which identify human pathogens, relevant mutations, antimicrobial resistance genes or allow for genotypic strain differentiation. Discussion will include a number of automated devices for template DNA preparation, amplification and characterization of specific amplicons which have been developed to standardize and economize the diagnostic workflow from receipt of a clinical specimen to reporting the corresponding PCR result. Moreover, the possibilities as well as the disadvantages of recent concepts for decentralized testing (POCT) will be discussed with the help of selected examples from routine practice in medical microbiology. Although the present technology can be considered to be robust and reliable for many routine purposes, further developments are ahead - and high resolution melting, multiplexing or high-throughput testing in microwell formats will definitely expand the spectrum of diagnostic applications in the months or years to come.

Real-time PCR Applications in the diagnostic of highly pathogenic viruses

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The detection of infectious agents is one of the most important tasks in the modern diagnostic laboratory. Many PCR-based methods for clinically relevant infectious agents have been implemented in routine diagnostics during recent years. However, general questions of biological safety and bio warfare have added an arsenal of agents to be identified in a rapid and reliable manner by the diagnostic laboratory. For those purposes, real-time PCR is the method of choice offering several benefits described extensively over the last years, including high specificity, speed and a significantly reduced risk of false positive results by carry over contamination. In addition to classical real-time PCR, various real-time PCR strategies and post-real-time PCR analytical techniques can be applied to meet the individual demands made by the diagnostic scenario. Certain situations may require the unambiguous identification of a virus type, as in others, a screening for virus families will be needed to give a first hint on the causative agent in a clinical sample, or even more important, in environmental samples. This presentation will give an overview over various approaches for individual problems that occur in the diagnostic of highly pathogenic virus.

Multiplex-PCR in clinical virology - benefits and limitations

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The ongoing discussion about the usefulness of multiplex-PCR in clinical virology is regularly driven by increasing technical possibilities, cost saving considerations and strong demands for shorter turn over times for testing. An intrinsic discrepancy between highest achievable sensitivity, robust performance and solid amplification conditions versus potential interference of a variety of components such as different primer and probes, increased background and potentially reduced sensitivity divides the community in the view of assessing the practical value of multiplex assays.
We exemplarily compared two commercially available multiplex detection kits: The „FTD respiratory pathogens“ 5-tube-multiplex PCR assay from „Fast-track Diagnostics“, which detects 15 different viral respiratory pathogens (three viral parameters in one tube using FAM-CY5-YAK/VIC-labeled probes) and also an internal control, and the „CMV HHV6,7,8 R-geneTMQuantification Kit“ (ARGENE). The second is not a „real-multiplex“ assay system in a narrow sense, since the different viral pathogens are detected in separate wells using fluorescent probes labeled with the same dye. However a general amplification program allows sensitive detection of multiple viral parameters in one run in many real-time cyclers. Compared to in-house assays, based on singleplex assays performed on either LightCycler 2.0 or ABI7500-fast platforms, both commercial kits render results similar in sensitivity and specificity to those generated by home-brew singleplex formats. As expected the interpretation of results generated with the „FTD respiratory pathogens“-kit requires a particular extent of attention. A complex analysis pattern and adjustment for the differently labeled probes in terms of variable dye intensities and amplification curve characteristics as well as individual control of each sample using the „component view“ is essential for a correct interpretation of the data. The use of an appropriate RT-PCR-amplification mix (not included in the FTD-Kit) is crucial for optimal results, as not all RT-PCR mixes tested yielded satisfactory results. We also tested the robustness of the multiplex kits, especially the CMV HHV6,7,8-assay, in terms of potential loss of efficacy in the presence of high levels of unspecific DNA. Patient samples often contain large amounts of host-cell RNA/DNA leading to reduced sensitivity. Addition of cellular RNA/DNA equivalent to 10E05 cells did not interfere with the herpes-viral-DNA detection. The usefulness of multiplex assays is largely dependent on the particular field of application. Distinct agreements between clinicians and laboratory regarding the handling of positive results for viral parameters actually not requested by the physician, clear-cut rules for reimbursement and arrangements about the range of necessary and essential viral analytes are indispensable prerequisites for a useful introduction of multiplex assays in the field of clinical virology.

Realtime PCR of bioterrorism agents

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The use of biological agents as a weapon has a long history. Besides ancient stories the so called industrial revolution and the growing knowledge in microbiology at the midth of the 19th century forcened some nations to develop and apply microorganism as a tactical weapon in wartime. It starts in the first world war and ended with the bioweapon programs of the USA and former Sowjetunion which were stopped 20 years ago. Todays threat of theses agents is based on criminal and socalled terrorism usage – mainly feared after the „911“-attack in the USA in 2001 and the subsequent acts involving weaponized anthrax spores. Since then potential biological terrorism agents, summarized in a threat list from the Centers of Disease Control (CDC, Atlanta, USA) including the classical „dirty dozen“, are the challenge to every country to ascertain the unambigious diagnosis of such agents. Traditional microbiological diagnosis relies on culture-based techniques that are time-consuming and can detect only culturable cells. However, recent developments in nucleic acid-based detection systems, in particular real-time PCR, offer significant advantages over culture-based methods for the detection of bioterrorism agents. Realtime PCR is therefore the cornerstone and gold standard in diagnosis according to specificity, sensitivity and speed. The problem is no longer the identification of such bugs but to guarantee the high quality standards when producing a result (e.g. Sensitivity,

Specificity, Internal control, Positive control, Stability, Validity, Reproducability). Because of the importance and medical and political consequences of such a bioterrorism attack PCR assays must be standardized to have the opportunity to confirm positive and/or negative results in different laboratories. Todays and future technical efforts and research are furthermore going to miniaturize („Nano-Chips"), fasten and automatise the different PCR-methods (e.g.„Biosensors").

Trans-renal DNA for infectious disease diagnosis

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The presence of DNA in urine is well established and can be utilised for the rapid and sensitive diagnosis of genito-urinary infections. A little over a decade ago the phenomenon of trans-renal DNA (trDNA) was described. This urinary nucleic acid originates outside the genito-urinary tract, as cell-free nucleic acids in plasma and serum (CNAPs) which pass through the kidneys into the urine where it can be detected. TrDNA has been used to verify foetal sex, foetal RhD status in RhD negative mothers, tumour marker identification, and to identify disease specific sequences in the urine of tuberculosis, malaria and HIV infected people. Urine is a particularly appealing clinical sample for the diagnosis of infectious disease due to the ease of donation, large volumes available, and reduced infection risk due to the absence of invasive sampling procedures. Despite the great potential of trDNA for disease diagnosis, the approach to detection has advanced little since its conception. An example is the detection of *Mycobacterium tuberculosis* (MTB) trDNA using conventional and one or two tube nested PCR, with visualisation of amplification products by gel electrophoresis. The detection of MTB trDNA in patients with pulmonary TB has widely varying sensitivities, with 7 % to 100 % reported in different studies. Recently, with an appreciation of the small size of some trDNA fragments, new approaches to amplification have been developed incorporating quantitative PCR. In addition to the size of the amplicons, we have addressed urinary DNA stability during storage, the DNA extraction method, and amplification target for detection of MTB trDNA using qPCR. All of these factors had an impact on detection sensitivity. Here we will discuss the application of qPCR to the detection of trDNA of infectious disease causing agents in urine and outline technical advances for improved sensitivity of detection.

New probes, same procedure - Improved results. Remove the false negatives and positives.

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Two new probesystems that can improve the outcome of Real-Time PCR assays and significantly reduce assay development time using standard procedures and equipment: EasyBeacons and HydrolEasy probes are presented togehter with their use in Real-time PCR. HydrolEasy probes are hydrolysis probes (TaqMan probes) modified with "pentabases" which increase specificity and signal-to-noise ratio over other known probes. EasyBeacons are nuclease resistant molecular beacon like probes, however without the need for designing a stem and with a broad working temperature. EasyBeacons can also be used for homogenous, end-point measurements. We will present examples of use and assay developments. One example is the development of an end-point detection assay using EasyBeacons detecting the presence of H274Y mutation in Bird Flu (H5N1) causing Tamiflu resistance. Likewise a Real-Time PCR assay for the corresponding H274Y mutation in human influenza (H1N1) causing Tamiflu resistance.
Other examples include the elimination of false positive signals in multiplex PCR reactions detecting airborne, infectious diseases and in cancer related SNP detections, increasing the sensitivity of detecting mutations in backgrounds of wild types and much more.

The use of DNA/RNA chimeric primers in qPCR for microbial detection and quantification

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The appearance of non-specific byproducts is an inherent problem in quantitative PCR assays resulting in an elevated detection threshold and reduced assay sensitivity. In order to overcome this problem we constructed DNA/RNA-chimeric primers and evaluated their use in qPCR. *Ehrlichia canis* and *Babesia canis* , two globally important tick-borne canine pathogens, were used as target pathogens in this study. Two different types of chimeric primers were constructed. In the first type, several RNA bases were incorporated into specific positions in the DNA primers while no RNA stretches were allowed. In the second type only one RNA base was incorporated to the 3' end of the DNA primer. PCR reactions were carried out without pre-amplification steps. The use of both types of chimeric primers resulted in a decreased undesirable byproducts formation and a ten-fold increase in the assay sensitivity. The affinity of chimeric junctions to DNA strands is usually lower than that of DNA base pairs. This phenomenon might be enhanced by chimeric primers, as the two annealed counterparts in primer-dimers are composed of chimeric junctions. It is apparent that chimeric primers provide a simple way to reduce the appearance of non-specific products in qPCR assays thereby increasing the assay's sensitivity and specificity.

Prevalence and viral load of oncogenic Human Papillomavirus types associated with cervical carcinoma in a population of North Italy

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A cross-sectional study was carried out in a population of North Italy to determine the prevalence of eight oncogenic human papillomavirus (HPV) types most commonly found in cervical carcinoma and to study the relationship between HPV DNA loads and severity of disease. A total of 597 cervical samples obtained from patients with pathological findings (n=472) and from women with normal cytology (n=125) were analyzed by means of normalized Real-time PCR assays to quantify HPV-16, -18, -31, -45 and -33 group (including -33, -52, -58, -67); the normalization of oncogenic HPV viral load was carried out by quantitation of a single copy gene. The two most common oncogenic HPV types found were 16 and 31 (24.3% and 22.9% of pathological samples, respectively); multiple infections were demonstrated in 22% of pathological samples. Overall, the HPV total viral load was found to increase with increasing severity of associated lesions, although a stronger association was observed only for HPV-31 and HPV-16 (γ=0.49 and 0.41, respectively) as compared to HPV-18 and -33 group (γ= 0.19 and 0.02, respectively). However, we found that high levels of HPV-31 or 33 group DNA could be prognostic of minor oncogenic risk for high-grade squamous intraepithelial lesions (H-SIL) (age adjusted odds ratio [AORs]= 1.57 and 1.26, respectively) than HPV-16 and HPV-18 (AORs = 30 and 8, respectively). The AORs also increased with HPV total viral load and reached a maximum of AORs= 15.7. Thus, HPV load is a type-dependent risk marker for the development of H-SIL.

Tuesday 10th March 2009

Session Diagnostics & Molecular Markers in agricultural & veterinary Science
Chair HHD. Meyer & U. Busch
Lecture hall HS 14

Rapid detection and differentiation of Campylobacter jejuni, C. coli and C. lari in food samples using a quadruplex real-time PCR assay

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Thermophilic Campylobacter species are the most common cause for food associated bacterial gastroenteritis worldwide. Therefore rapid and reliable methods for their detection and differentiation are required. A quadruplex real-time PCR assay based on probes for simultaneous detection and®four differently labelled TaqMan differentiation of C. jejuni, C. coli and C. lari was established and validated in food products. It combines two previously published PCR assays for C. jejuni and C. coli with a newly developed detection assay for C. lari and an internal amplification control system. Each assay is based on another gene. The selectivity of the method was determined by analyzing 70 Campylobacter strains as well as 43 strains from other bacterial species. The

sensitivity was 50 fg DNA of C. jejuni and C. lari, respectively, and 500 fg DNA of C. coli per PCR reaction. It was possible to detect 1 to 10 cfu per 25 g food prior to pre-enrichment of all three species. The assay showed a high degree of precision and robustness. More than 400 various food samples (poultry, mussels, fish, meat) were analyzed after 48 hours of pre-enrichment parallel to the conventional diagnostic method including cultural detection and biochemical identification of the species. The established real-time PCR detected more positive samples (55.4%) than the conventional method (40.3%). In terms of species identification, the quadruplex real-time PCR detected a high percentage of mixed infections (32.6%) analysing poultry samples which is of epidemiological interest. The analysis of a portion of the samples (n=147) after only 24 hours of enrichment using the quadruplex real-time PCR resulted in the detection of 98.0% of the cultural positive samples and 80.8% of the PCR positive samples after 48 hours of enrichment. By providing reliable results within two days compared to 7-8 days for the conventional diagnostic method, the presented quadruplex real-time PCR is a good alternative to accelerate routine analysis and therefore enhance food safety.

The use of transcriptomics for biomarker development to trace anabolic hormone functions.

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The combat against the misuse of growth promoting agents is a major topic in agricultural meat production. In routine screening, hormone residues of all known growth promoting agents are detected by immuno assays or chromatographical methods in combination with mass spectrometry. To overcome the detection by these routine screening methods new xenobiotic growth promoters and new ways of application were developed, e.g. the combination of different agents in hormone cocktails are employed. To enable an efficient tracing of misused anabolic substances it is necessary to develop new screening technologies for a broad range of illegal drugs including newly designed xenobiotic anabolic agents. A promising approach to develop such a new screening method is expression profiling of validated marker genes to discover the misuse of anabolic hormones by indirectly detecting their physiological action. In an animal trial with 9 Nguni heifers, treated with the anabolic combination trenbolone acetate plus estradiol, gene expression changes in blood, liver and vaginal epithelial cells were quantified by qRT-PCR. In all three tissues several hormone responsive genes were identified. To verify these expression changes and to select them as first biomarker candidates different biostatistical tools are available. Principle Components Analysis and Hierarchical Clustering (GenEx Ver. 4.3.6, MultiD, Gothenburg, Sweden) were successfully used to demonstrate the potential of the transcriptomic approach for the development of a new screening system to detect the misuse of anabolic agents in cattle.

Development of a highly sensitive and specific assay to detect Staphylococcus aureus in bovine mastitic milk

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1. Setting and aim of the study - Bacteriological analysis of milk on agar plates is still the gold standard for the microbiological diagnosis of *Staphylococcus aureus* (*S. aureus*) intramammary infections. However, it often provides unsatisfactory results. Using aliquots of 100µl per sample, the overall bacteriological sensitivity is 75% but may vary between 41% and 100% for individual cows. Under routine conditions with smaller aliquots (10µl), the results are even worse. Only when 3 consecutive milk samples are taken, the sensitivity improves to 98%. This procedure, however, is expensive and is not satisfactory for clinical purposes. To improve the diagnostics for *S. aureus* in mastitic milk, we developed a test that allows the highly specific detection of S. aureus in bovine milk samples at very low concentrations. 2. Material and methods - An assay was developed which is based on a novel, fast and patented procedure to prepare S. aureus from milk processing 400µl of milk. This step is followed by DNA extraction and real-time quantitative PCR (QPCR) with a *S. aureus* -specific gene as a target (*nuc* gene). QPCR was performed with 2 primers and a fluorescent probe. An internal control DNA was included so that negative results of target gene amplification could be interpreted. The QPCR method was compared to classical bacteriology using 77 clinical milk samples. Bacteriological analysis of each sample was done according to the guidelines of the National Mastitis Council using 100µl-aliquots. 3. Results - For the clinical milk samples, the analytical sensitivity of the novel assay was 500 to 2000 times higher than conventional routine bacteriology. The diagnostic specificity was 100% and was not influenced by contaminating DNA extracted from various *Staphylococcus* spp. The test is further characterized by a low intra- and inter-assay variability as well as by a very good recovery (94%). Furthermore, a high correlation ($r = 0.925$) between the agar plate counts and the QPCR methodology over the whole range was found. In addition, our test revealed considerably more positive results than bacteriology ($p < 0.001$). Finally, the novel assay is fast as a definite result is obtained within 5 hours including bacteria preparation, DNA extraction and QPCR. 4. Conclusion - We developed a novel QPCR-based assay to detect and quantify *S. aureus* in milk. It is characterized by excellent diagnostic properties such as a very high analytical sensitivity and specificity. Due to its distinctive features, the assay is expected to become an important diagnostic tool in the context of bovine mastitis caused by *S. aureus* .

Development of a real-time PCR Method for Detection and Quantification of the Fungal Biocontrol Agent Trichoderma atroviride SC1 in Soil

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Trichoderma (Hypocreales, Ascomycota) is a widespread genus in nature and several Trichoderma species are used in industrial processes and as biocontrol agents against crop diseases. Accurately monitoring the persistence and spread of microorganisms released into the environment is crucial. Real-time PCR methods for genus/species/strain identification of microorganisms are currently developed to overcome the difficulties of classical microbiological and enzymatic monitoring methods. The aim of the present study was to develop and validate a specific real-time PCR-based method for detecting *Trichoderma atroviride* SC1 (Patent deposit PCT/IT2008/000196) in soil, according the common criteria of the validation process (i.e. specificity, applicability, sensitivity, dynamic range and accuracy). The strain specificity of this method was achieved through the use of a TaqMan probe constructed on two base pair mismatches in the sequence of the first intron in the endochitinase 42 gene (ech42). The two mismatches inside the probe were sufficient for discriminating against different *Trichoderma* and other fungal strains, even in situations in which those mismatches were the only differences between two strains. The calculation of the LOD and the LOQ is based on the increase in relative standard deviation (RSDr) that corresponds to the decrease in the number of genome copies. This procedure is the same one used to detect and quantify the contamination of genetically modified organisms and, therefore per analogy, should be suitable for the monitoring of other microorganisms in the environment, as well (European Network GMO Laboratories, 2005). Thresholds were set at 33% and 25% of the RSDr for the quantitative methods. The absolute LOD (95% of the amplified samples) was also calculated, and this showed that the two approaches (RT-PCR and Colony Forming Units counting) gave similar values. With this calculation, we postulate that a CFU density above 0.6-0.7*104 g soil-1 can certainly be detected and a density above 2.2-3.4*104 CFU g soil-1 can also be quantified. Lower inoculum doses can also be detected and quantified, but our data show that the relative standard deviation increases dramatically at these inoculum levels and that some values within a repetition will be zero. The accuracy, defined as the correlation between the values obtained with the two methods (Genome Copies versus CFU), drops when genome copy numbers are below the LOQ and becomes erratic when genome copy numbers are below the calculated LOD. We confirmed its validity in monitoring and quantifying the presence of T. atroviride SC1 after its release in microcosms under controlled conditions and in the environment. This procedure has a low throughput time and allows the analysis of many soil samples within a short time period. These results suggest that this method can be successfully used to trace the fate of *T. atroviride* SC1 when applied as Biocontrol Agent in the environment.

Temperature influence on expression of selected genes in Mycobacterium avium subsp. paratuberculosis in milk environment

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Mycobacterium avium subsp. *paratuberculosis* (*MAP*) is characterised as Gram positive and acid-fast bacterium. *MAP* has been known as a causal agent of Johne´s disease, a chronic inflammation of the intestine in ruminants, thus it has a great economic negative impact on health, economy and breeding. The continued increase of incidence for resistant *MAP* in milk and its products seems to be possible problem for human healthcare, because *MAP* was admitted as one of triggers causing Crohn's disease in humans (a chronic inflammatory disease especially of small intestine). Consequently, some methods of milk pasteurization are not sufficient to kill all *MAP* cells present in milk and *MAP* has been cultured from raw or pasteurized milk and isolated from cheese. The aim of this work was to found out if the expression of 22 candidate genes of *MAP* genes could be influenced by its incubation in milk and yoghurt spiked by *MAP* . Different temperature treatments of milk samples containing *MAP* were applied to mimic pasteurization conditions in diary industry.
Two bovine isolates (8672 and 8819) from clinically manifested cows and one isolate (12146) obtained from cow without PTB symptoms were included in this study. Candidate genes were chosen according to their connection with *MAP* virulence, adhesion and stress response. Relative quantification of *MAP* mRNA using quantitative real-time reverse transcription (RT)-PCR was used. The results obtained from *MAP* cultivation in milk and yogurt samples under different temperature treatments showed quite similar gene expression pattern. *Mmp* gene coding 35kDa major membrane protein and *modD* coding fibronectin attachment protein (FAP-P) were the mainly up-regulated genes generally at all tested isolates. The above mentioned up-regulation was observed mainly after the influence of higher temperatures, rather than 65°C. Down-regulation under all temperature treatments for all tested strains was observed in case of *Mce3* gene, which was identified as important for invasion and survival within macrophages.

Applying Real-time PCR to Determine Co-dominant Genotypes of Dominant SCAR Markers in Common Bean

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Many dominant SCAR markers have been developed that are linked to disease resistance genes in common bean (*Phaseolus vulgaris* L.). Unfortunately, these dominant markers cannot be used to distinguish between homozygous or heterozygous genotypes. To maximize the likelihood of identifying F2 plants that are fixed for the resistance alleles it is necessary to self pollinate plants that amplify a linked SCAR marker and conduct progeny tests for disease resistance. The need to perform progeny testing to identify homozygous plants delays progress in improving disease resistance and increases costs associated with resistance breeding. We have developed a method using real-time PCR to co-dominantly interpret the genotypes of bean plants for several dominant SCAR markers. Remnant F1 plants are used as a comparative reference sample and segregant plants are genotyped based on their real-time PCR results relative to probability distributions determined for heterozygotes. In the case of the resistance genes, I and bc-12, which condition resistance to bean common mosaic virus (BCMV), real-time PCR assays were developed for SCAR markers that were linked to each resistance gene. A multiplex PCR reaction could unambiguously assign F2 plants to one of nine genotypes. F2 plants also were genotyped for the I and bc-12 alleles by performing F3 progeny tests for virus resistance. Agreement between the two methods was 100% (198/198) for the bc-12 allele, and 92.4% (183/198) for the I allele.
Resistance to common bacterial blight (CBB) in common bean is quantitatively inherited. We examined the interaction between two independent QTL, BC420 and SAP6, using a near isogenic (NIL) population. Dominant SCAR markers linked to both QTL were interpreted as codominant markers using real time PCR assays. Reaction to CBB in NILs was characterized by an epistatic interaction between BC420 and SU91 such that: i) the expression of BC420 was epistatically suppressed by a homozygous recessive su91//su91 genotype; ii) SU91//SU91 and SU91//su91 genotypes conditioned an intermediate disease reaction when homozygous recessive for bc420//bc420; and iii) the highest level of disease resistance was conferred by genotypes with at least a single resistance allele at both QTL (BC420//-; SU91//-). This is consistent with a recessive epistatic model of inheritance between two loci. These results clearly demonstrate that real-time PCR assays provide a robust method for genotyping seedlings and in some cases may eliminate the need for progeny testing, which will reduce costs and accelerate progress in breeding programs. In addition, the ability to co-dominantly interpret plant genotypes for SCAR markers may improve understanding of how QTL interact to condition disease resistance.

Comparison of AOE activities and expression levels in the kidney during the development of hypertension in SHR

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Oxidative stress has been implicated in essential hypertension. However, the precise redox status, in particular, protein and mRNA levels of antioxidant enzymes (AOE) and the activities, during the pathogenesis of hypertension remains inconclusive. This study attempts to determine these for some of the AOE during the development of hypertension in spontaneously hypertensive rats (SHR). Body weight and systolic blood pressure was measured in 2, 4, 7, 8, 10, 12 and 16-week old SHR and age-matched WKY rats (n = 6 per group). After the measurement of blood pressure, rats were sacrificed and the kidneys were collected for measurement of activities of SOD, GPx and CAT. CuZn-SOD, Mn-SOD and GPx mRNA levels were determined at 4, 7, 8, 10, 12 and 16 weeks of age by relative quantification via reverse transcriptase Real-time PCR. CAT protein levels were determined at 4, 8, 12 and 16 weeks by Western Blot analysis. Data were analysed using ANOVA and a 'p' of < 0.05 was considered statistically significant.
Systolic blood pressure was significantly higher from the age of 7 weeks while body weight was significantly lower in all SHR groups when compared to their age-matched WKY rats. SOD enzymatic activity and mRNA levels of CuZn-SOD and Mn-SOD were not different between the SHR and WKY rats. GPx activity was significantly lower from 4 weeks of age onwards, while CAT activity was significantly higher from 2 weeks of age onwards in the SHR when compared to age-matched WKY rats. CAT protein levels were significantly higher from 8 weeks of age onwards in the SHR when compared to WKY rats. GPx mRNA levels were lower at 7, 8, 10 and 16 weeks, being statistically significant in rats at 8 and 10 weeks of age. Interestingly, total antioxidant status (TAS) was higher in SHR throughout the period of study, being statistically significant at 4, 10, 12, and 16 weeks when compared to age-matched WKY rats. TBARS level was only found to be significantly higher in the SHR at 16 weeks. Although TAS and levels of TBARS do not indicate the presence of oxidative stress during the onset of hypertension in the SHR, the altered pattern of CAT and GPx activities, together with levels of CAT protein and GPx mRNA, nevertheless suggest an altered redox status in SHR, which might or might not be responsible for the hypertension in the SHR, and it clearly needs further study.

A quantitative real-time PCR assay for Ehrlichia ruminantium using pCS20

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Heartwater is a tick borne disease that affects ruminants and wild animals in Africa south of the Sahara. It is caused by *Ehrlichia ruminantium* and transmitted by the tick Amblyomma hebraeum. The protocols currently used to detect heartwater take several days to complete. Here we describe the development of a pCS20 quantitative real-time PCR TaqMan probe assay to detect _E. ruminantium _in livestock blood and ticks from the field. The assay is based on the conserved pCS20 gene region of *E. ruminantium* that contains two overlapping genes, rnc and ctaG (Collins et al., 2005). The pCS20 quantitative real-time PCR TaqMan probe was compared to the currently used pCS20 PCR and PCR/32P-probe

test with regards to sensitivity, specificity and the ability to detect DNA in field samples and in blood from experimentally infected sheep. This investigation showed that the pCS20 quantitative real-time PCR TaqMan probe was the most sensitive assay detecting 7 copies of DNA/µl of cell culture. All three assays however, cross react with *E. canis* and *E. chaffeensis* . The pCS20 real-time PCR detected significantly more positive field samples. Both the PCR and pCS20 real-time PCR could only detect *E. ruminantium* parasites in the blood of experimentally infected sheep during the febrile reaction. The PCR/32P-probe assay however detected the parasite DNA one day before and during the febrile reaction. Thus because this new quantitative pCS20 real-time PCR TaqMan probe assay was the most sensitive and can be performed within 2 h it is an effective assay for epidemiological surveillance and monitoring of infected animals.

Relative gene expression of acid-inducible genes in acid-adapted Escherichia coli O157:H7 during lactoperoxidase and lactic acid challenge

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Acid inducible cross-protection facilitated by the alternate sigma factor, RpoS is glucose repressed in acid-adapted Escherichia coli O157:H7. Several studies have used minimal media to characterize acid adaptation and acid resistance in E. coli O157:H7. However food systems themselves are complex and in many cases have glucose present. There is insufficient characterization of cross-protection of acid-adapted E. coli O157:H7 during the actual environmental stress. Cell counts of acid-adapted cells challenged to the combination of activated lactoperoxidase (LP) and lactic acid at pH levels of 4.0 and 5.0 in Tryptone Soy Broth (TSB) indicated that acid-adapted cells were cross-protected against activated LP and low pH treatments. To better understand the association between glucose repressed RpoS and cross-protection in E. coli O157:H7 against activated LP and lactic acid, this study investigated the relative expression of selected acid-inducible genes in acid-adapted E. coli O157:H7 challenged to lactic acid (pH 4.0) and activated LP in TSB using quantitative Real Time-PCR. Relative expression of acid inducible genes indicated increased expression of glutamate decarboxylase gene gadA and outer membrane porins ompC and ompF, however rpoS expression was not significantly increased in acid-adapted E. coli O157:H7 cells challenged to activated LP at pH 7.4, suggesting that rpoS may have been repressed due to the presence of glucose. Increased expression of the magnesium transporter gene corA in non-adapted E. coli O157:H7 challenged to activated LP only, indicates its involvement in LP resistance at pH 7.4. However, corA was not acid inducible and it did not contribute to cross-protection at low pH. All acid-inducible genes tested in this study were down regulated in E. coli O157:H7 cells challenged to pH 4.0 only and pH 4.0 in combination with activated LP. Although acid-adaptation greatly enhanced survival of E. coli O157:H7 to lactic acid (pH 4.0) and activated LP treatments, the decreased expression of acid inducible genes tested suggests that at lethal pH levels, E. coli O157:H7 shuts down expression of new acid-inducible genes, a process that may otherwise lead to metabolic exhaustion in a lethal environment. It is however likely that proteins expressed during acid-adaptation were still active in protecting the acid-adapted cells against lethal pH and antimicrobial damage at the physiological level.

A QUANTITATIVE PCR TECHNIQUE FOR EVALUATION OF ARSENIC MOBILITY IN HEAVY METAL CONTAMINATED SAMPLES

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Quantitative PCR is a powerful and sensitive method for monitoring microbial processes in the environment. The purpose of our study was quantification of two target genes in gold mine samples, highly contaminated with arsenic, in order to study microbial arsenic remediation. We quantified *arsC* and *aroA* , which are responsible for arsenate reduction and arsenite oxidation respectively. Samples from the Con Mine (Yellowknife, Northwest Territories, Canada) were collected from multiple depth levels down to 1.6 km, with detected amounts of arsenic from 48 to 2781 ppb depending on depth. A major challenge for these qPCR experiments was preparing PCR-suitable DNA directly from mine samples. The preparation process needed to remove mine contaminants that inhibit enzymes involved in the amplification process. These samples contained not only extremely high level of total arsenic, but also had high salinity and high amounts of other heavy metals. Several different methods to prepare PCR-suitable DNA from heavy metal containing soil were assessed, and a new DNA extraction protocol was developed for the Con mine samples. The samples with the highest arsenic content from each level were chosen for real-time PCR analysis for the *arsC* and *aroA* genes. For the *arsC* gene three sets of primers were designed, and for the *aroA* gene two sets of published primers were used. Dark red soil samples from 1.5 km depth with a concentration of total arsenic from 1688 to 1874 ppb showed the highest level of *aroA* gene copy numbers in the range of 1350-9560 per ng of total DNA. The *arsC* gene was present in the pure strains of several *Bacillus* species isolated from the 1.07 km depth level. However, the highest detected *arsC* copy numbers in the DNA obtained from mine samples were much lower than those for *aroA* (maximum 10 copies per ng of DNA). The predominance of arsenite-oxidase genes in the Con mine samples suggests a high activity of bacteria converting arsenite (AsIII) to the less mobile arsenate (AsV). The method proved to be sensitive; however, the results of the PCR-derived quantifications must be interpreted cautiously, and consideration should be given to the fact that the primers designed do not include all variations of the arsC and aroA genes present in the mine biota. Quantification can also be compromised by formation of even a small amount of primer-dimers. Nevertheless, as we have demonstrated, qPCR is a useful tool for monitoring of arsenic mobility and these results set the stage for a better understanding of the distribution and role of arsenite-oxidizing microbial activity in an environment.

Use of real-time PCR for detection of bovine herpesvirus-1 in cattle and buffalo frozen semen

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Bovine herpesvirus-1 (BHV-1) is the causative agent of infectious bovine rhinotracheitis (IBR) / infectious pustular vulvovaginitis (IPV) in bovines. Cattle and buffaloes infected with BHV-1 remain lifelong carrier and shed the virus intermittently in semen. In the present study attempts were made to validate a real-time PCR protocol to screen cattle and buffalo frozen semen for BHV-1. The gB gene (97bp) of the virus was amplified (OIE, 2008) using TaqMan chemistry in BioRad iCycler.

A part of gB gene of an Indian isolate of BHV-1 virus was amplified by PCR and cloned into pDrive cloning vector to derive a positive control. The positive construct was confirmed by nucleotide sequencing and restriction enzyme analysis. A linear increase in the cycle threshold (ct) value ranging from 15.2 to 39.4 was observed upon serial tenfold dilutions of the positive control DNA construct from 1X10^10 to 1X10^1 molecules per reaction, respectively for real-time PCR. No amplification was recorded in PCR reaction with known negative control, showing the ct value above 40. Hence, the samples showing a ct value of <40 was considered as positive and >40 was considered as negative. Further, viral DNA was extracted from serially diluted BHV-1 infected cell culture supernatant and 5ul each of DNA preparation was used in real-time PCR for amplification. A positive reaction could be recorded in highest dilution of virus containing 10 ^0.7 TCID50/50ul.

After validation of the test, a total of 184 batches of frozen semen collected from cattle and buffaloes were inoculated in cell culture (MDBK) for virus isolation and tested by real-time PCR. In cell culture 16 (8.69%) batches turned positive for BHV-1. But by real-time PCR 32 (17.39%) were declared positive for BHV-1 viral genome. The cell culture isolated viruses were confirmed as BHV-1 by conventional PCR and micro serum neutralization test. In comparison to virus isolation, sensitivity and specificity of real-time PCR were found to be 100 and 91.37 per cent, respectively.

To examine the test-precision of this PCR protocol, all the DNA samples from 184 semen batches were tested for amplification in duplicates at the same time. The mean ct value of the positive samples in two reactions was 36.44 and 36.30 with an SD and CV of 0.10 and 0.27, respectively. To determine inter assay variability of real-time PCR, 50 random DNA samples out of 184 batches of

frozen semen were again amplified at different time periods in duplicate. The mean ct values for the positive samples in first and second run were 35.35 (SD 0.68) and 36.09 (SD 0.45), respectively indicating the reproducibility of the test.
Some representative batches of frozen semen, declared negative by real-time PCR in two consecutive runs were chosen and spiked with BHV-1 virus which turned positive by real-time PCR indicating the negative semen batches are true negative and were devoid of PCR inhibitory compound. This study indicates that real-time PCR could be useful to screen frozen semen for BHV-1.

Viability of Mycobacterium avium subsp. paratuberculosis as measured by PMA-F57real time qPCR
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Mycobacterium avium subspecies *paratuberculosis* (*MAP*) is a pathogen which causes chronic granulomatous enteritis known as paratuberculosis or Johne's disease. Its primary hosts are domestic ruminants. Clinical signs are intermittent diarrhoea, loss of weight, decreased milk production and ultimately death of the infected animal. It is suspected to be one of the triggers of Crohn's disease in humans that exhibits similar symptoms as in cattle. But Crohn's disease is autoimmune disorder and the theory of the disease origin does not assume presence of viable MAP cells. As the main vehicles of the *MAP* transfer of humans are considered foodstuff from cattle. *MAP* was previously detected in milk and milk products and also in meat. It was also reported previously that *MAP* can survive common pasteurization processes.
For the detection of *MAP* in different samples, the cultivation is considered to be a gold standard. This technique exclusively can detect only viable *MAP* cells. Other techniques as PCR derived methods can detect all *MAP* despite of the viability. Due the fact that culture of *MAP* requires at least 3 months there is a need for the more rapid technique for the determination of the *MAP* viability.
In our work we have introduced and standardised the method for the *MAP* viability profiling based of the usage of propidium monoazide (PMA) and subsequent real time qPCR analysis. PMA incorporates to the DNA of dead cells and by this bind it disables the DNA polymerase to elongate such modified DNA strand. By the analysis of PMA treated and untreated samples, it can be concluded what portion of viable and dead cells is present in the sample.
We have exposed two different *MAP* isolates (collection strain CAPM 6381 and field isolate 8819) to temperatures from 60°C to 90°C for 30 seconds to determine the viability after the heat shock in time. After the heat treatment, the *MAP* cells were put to a liquid media and the viability and total amount of the *MAP* cells were measured weekly by *F57* qPCR. The data suggest that *MAP* cannot survive 70°C for 30 seconds, which is in concordance with previously published results. The newly introduced method is reliable and faster and in comparison with cultivation it can determine not only the viability of *MAP* , but also the total amount of *MAP* cells.
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Tuesday 10th March 2009

Session RNAi: microRNA – siRNA Applications
Chair M. Castoldi & MW. Pfaffl
Lecture hall HS 15

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miQPCR: A novel approach for expression profiling of mature microRNAs.
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Recent studies indicate that dysfunctional expression of miRNAs is implicated in a range of human pathological conditions, including heart disease, neurological disorders and cancers. The latter role suggests that miRNAs may serve as biomarkers for diagnosis and prognosis of human diseases, emphasizing the importance of studying miRNAs expression and function. Here we present "miQPCR", a novel approach for quantitation of miRNAs expression by using real-time PCR. Important advantages of this method are; i) it performs a 'one-step' reverse transcription of all small RNAs contained in the sample, ii) it is specific for mature miRNAs, iii) it allows the Tm adjustment of miRNA specific primers and iv) it is cost effective. In summary, miQPCR is a robust method suitable for the highthroughput detection and specific quantification of mature miRNAs by using RT-PCR.

An inflammatory microRNA signature in muscle cells - a comparative study of cellular models and technological platforms.
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Elevations in circulating inflammatory cytokines, particularly TNF-α in chronic disease conditions like cancer, AIDS, and inflammatory or non-inflammatory myopathies are associated with muscle wasting and cachexia. Nuclear factor kappa B (NF-κB), a downstream effector of TNF-α, forms a causal link to the inhibition of muscle differentiation by negatively regulating the myogenic transcription factor MyoD. Muscle specific microRNAs play important roles in skeletal muscle proliferation and differentiation and have been reported to be regulated by MyoD and other myogenic regulator factors. Based on this link between TNF-α and muscle specific microRNA expression the hypothesis that muscle specific miRs are potent mediators of TNF-α signalling involved in impaired muscle regeneration was investigated. A comparative study using global profiling and individual analysis of microRNA expression provides experimental evidence for the proposed specific influence of TNF-α on muscle specific microRNA expression in differentiating muscle cells.

Discovery and Validation of Novel Human MicroRNA Genes by SOLiD and TaqMan
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MicroRNA (miRNA) has been quickly identified as an important regulator of gene expression. Dysregulation of miRNA is often associated with diseases like cancer. A total of 695 human miRNA genes have been identified to date. Many researchers believe that hundreds of additional novel human miRNAs are yet to be discovered. Conventional cloning and Sanger sequencing methods may not achieve this increasingly difficult task. Evidence suggests that unknown novel miRNAs may be present in only a specific cell type, at a particular developmental stage, or relatively low expression levels. We have developed a novel approach to construct libraries of small RNA sequences. This method enables the simultaneous and directional ligation of adaptors to the ends of RNA as the first step in the construction of small RNA libraries suitable for the 'Sequencing by Oligonucleotide Ligation and Detection' (SOLiD (TM)) platform. We constructed and sequenced ten small RNA libraries from ten different human tissues, generating ~200 million tags of 25 or 35 bases. Analysis of this data demonstrated detection of up to six logs of dynamic range and correlation to real-time PCR, indicating its capability of deep sequencing as well as profiling small non-coding RNAs. Using an *in silico* 'Support Vector Machine' (SVM) algorithm, we developed a method to predict novel miRNA-like sequences present in the various tissue samples and found few hundreds of potential novel miRNAs. Using a subset of these novel sequences we demonstrate the validity of over 50% of them using custom TaqMan® Small RNA Assays. These results demonstrate a simplified, quantitative, and sensitive methodology for both profiling and discovery of small non-coding RNA targets, including

miRNAs, using SOLiD. Furthermore, Custom TaqMan® Small RNA Assays can be used for follow-up screening and validation to identify novel miRNAs related to specific diseases.

mRNA & microRNA integrity - the key to success

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The assessment of RNA integrity is an important step in obtaining meaningful mRNA and microRNA expression results. Degraded RNA may strongly compromise the experimental data of quantitative downstream applications, like real-time RT-PCR, RT-qPCR arrays, and microarrays. Using integer mRNA and microRNA is one major key element for the reliable and successful quantification of nucleic acids . To verify RNA quality nowadays automated capillary-electrophoresis systems are available which are on the way to become the standard in RNA quality assessment, e.g. Agilent Bioanalyzer & Bio-Rad Experion. Both platforms determine RNA quality either by using the ribosomal 28S/18S ratio, or a numerical system which represents the integrity of RNA, called RIN or RQI respectively. Profiles generated yield information on absolute or relative RNA concentration, allow a visual inspection of RNA integrity, and in terms of total RNA, both generate approximated ratios between the mass of ribosomal subunits. Herein, the importance of overall RNA integrity was analyzed by determining the mRNA and microRNA quality of different bovine tissues after various extraction procedures, and at 11 different decreasing total RNA degradation levels. We put a special focus on blood, because it is the most important tissue source in molecular diagnostics. Independent quantitative and qualitative analysis systems are compared (OD measurement, NanoDrop, Bioanalyzer 2100 and Experion) and the advantages and disadvantages are shown. Further the significant comparison and correlation between the total RNA integrity on RT-PCR performance on mRNA and microRNA is described. On the basis of the derived results we can argue that real-time RT-PCR performance on mRNA and microRNA level is significantly negative correlated with the RNA integrity. We can recommend a RIN/RQI values higher than five as good over all RNA quality and values higher than eight as perfect suitable for quantitative downstream applications.

microRNAs - developing new tools for diagnostics - Join forces with IMGM Laboratories to make your miRNA project a success

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microRNAs (miRNAs) represent a recently discovered class of small, non-coding RNAs which are involved in gene expression and play a critical role in many biological and pathological processes. In the last few years, miRNAs have emerged as major players in the complex networks of gene regulation and have therefore gained high interest as a new class of clinically relevant biomarkers. As biomarkers, miRNAs have a clear advantage over mRNAs. They provide an excellent detectability due to their stability in vivo, in vitro, in formalin-fixed and paraffin-embedded tissues (FFPE) as well as in blood and other body fluids. However, the detection of miRNAs presents a great technical challenge due to their shortness, their high degree of sequence homology, their partly high copy number and the lack of a poly(A) tail.
Microarrays and quantitative real-time PCR, all proven technologies from the field of gene expression, have specifically been adapted for miRNA analysis. The standardized use of these technologies in miRNA research will make it possible to identify individual miRNAs as well as complete miRNA signatures with a high degree of accuracy. This approach has already been used with very promising results in the following fields of applied medicine – in cancer diagnostics, in the development of miRNA-based therapeutics and for the prognosis of therapy success. Since the first report on the direct involvement of miRNAs in cancer, the combined scientific efforts have already led to a significantly improved understanding of carcinogenesis (cf. Calin & Croce 2007, Mallardo et al. 2008, Sassen et al. 2008). As a result, the first diagnostic cancer drug resistance tests have recently been announced by Oncotech (now owned by Exiqon) and Rosetta Genomics in collaboration with the Columbia University Medical Center. Further screening test are planned to be released soon.
One of the major challenges is the development of novel miRNA targeting therapies based on the knowledge about disease specific miRNA expression patterns. Due to the multi-gene-targeting characteristic of this promising new class of small RNAs, broad functional analyses with a focus on global gene expression will have to be carried out.
The use of modern miRNA analytics in the field of applied medicine is one of the hottest research topics in the pharmaceutical industry. Development in the coming years will show whether miRNA-based products will find broad application in diagnostics, therapeutics and prognostics. It will be interesting to see whether and to which extent miRNA research will play a significant role in improving the human condition. Standardized workflows combined with technical expertise, as offered by the accredited service provider IMGM Laboratories GmbH, make it possible to carry out miRNA research in a fast and straightforward fashion.

A novel and universal method for microRNA RT-qPCR data normalization

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Gene expression analysis of microRNA molecules is becoming increasingly important in biological research. Although several quantification strategies have been developed, real-time quantitative RT-PCR is turning out to be the method of choice to measure microRNA expression levels, both in small and large scale profiling studies, due to its unprecedented sensitivity, specificity and dynamic range of linear quantification. However, the accuracy of the results is largely dependent on proper data normalization of the data. While current strategies rely on the use of endogenous small RNA controls, thorough evaluation and validation is lacking. In this study we assess the use of the mean expression value of all expressed microRNAs as a normalization factor and compare its performance to the currently adopted approach.
Through the analysis of high dimensional microRNA expression profiles from 147 samples in 5 different tissue groups (neuroblastoma, T-ALL, leukemia with EVI1 overexpression, normal bone marrow, pool of normal adult tissues), we demonstrate that normalization using the mean expression value outperforms the use of small RNA controls in terms of better reduction of technical variation. Our method, allows for a more accurate appreciation of biological changes, as illustrated by differential miR-17-92 expression in MYCN amplified neuroblastoma tumour samples. Finally, we propose a workflow for small scale microRNA profiling studies which is based on the selection of microRNAs that resemble the mean expression value and thus mimic mean expression value normalization.
The proposed strategy is both straightforward and universally applicable and results in a more reliable assessment of biological microRNA variation.

Highly sensitive and specific LNA™-enhanced real-time PCR for microRNA expression analysis

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MicroRNAs (miRNAs) comprise a family of highly conserved small non-coding RNAs (~22 nt). As regulators of post-transcriptional gene expression, miRNAs play an essential role in many parts of development, differentiation, and physiological processes. It is now established that altered miRNA expression profiles are associated with a number of different diseases including heart disease, neurological disorders and human cancers. This suggests the use of miRNAs as a novel class of biologically important biomarkers for disease diagnosis and prognosis. The study of the expression and functional effects of miRNAs is complicated by their small size and limited availability of sample. We here present a highly sensitive real-time PCR method for quantification of miRNAs. One of the advantages of the Locked Nucleic Acid (LNA™) technology is that very short high-affinity miRNA-specific primers can be designed to work under general PCR conditions. The LNA™ primer design enables a simple and robust two-step method employing two different miRNA-specific primers: a miRNA-specific RT primer is employed in the first-strand cDNA synthesis, and for the following

SYBR® Green-based quantitative PCR detection, an LNA™-enhanced primer targets the miRNA sequence at the opposite end. Hence, the method offers accurate quantification of specific miRNAs directly from total RNA. The LNA™-based assays show a high dynamic range with a linear readout of miRNA concentrations spanning more than 8 orders of magnitude, enabling detection of as few as 10 RNA copies.
With proper normalization using stably expressed microRNAs or other small non-coding RNAs, we have seen differential microRNA expression between different cell lines which shows an excellent overall correlation between differences detected on the miRCURY LNA™ microRNA Arrays and those detected using real-time PCR. This confirms that miRCury qPCR can be used for detection of microRNA biomarkers, and advents a future use in diagnostics.

Quantification and Functional Analysis of miRNA in Mammalian Cells
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We have developed a robust and accurate method for transcriptome-wide miRNA quantification using SYBR Green detection-based, real-time PCR. The miScript PCR System is highly specific, sensitive, and requires very small amounts of input RNA. The system enables detection of miRNAs as well as mRNAs using the same cDNA preparation allowing simultaneous quantification of miRNAs and target mRNAs. The system also allows detection of non-coding RNAs other than miRNA. Application of this technology to miRNA expression profiling in a model human cell-culture system will be demonstrated. The use of the miScript PCR System in combination with new QIAGEN tools for miRNA functional analysis tools in mammalian cells will also be discussed.

A Novel Simple and Inexpensive Assay for microRNAs Detection
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The most commonly used assay for microRNA (miRNA) detection and quantification is quantitive real time PCR (qRT-PCR) that is based on either SYBR Green or TaqMan probe. Since SYBR Green intercalates into double-stranded DNA in a non-specific manner, it commonly detects non-specific PCR products and primer-dimers, leading to false-positive results. In contrast, TaqMan probe based assays are highly sensitive and accurate. However, each assay requires an individual miRNA-specific fluorescent probe, making this technique expensive and not optimal for the analysis of a large number of miRNAs. On this background we have developed a novel cost effective real-time PCR method based on the use of a universal probe library (UPL, Roche) that enables the detection and quantification of miRNAs with high sensitivity and specificity. The assay consists of the three following steps: a) Preparation of cDNA libraries by polyadenylation of total RNA. b) Reverse transcription of the cDNA using a universal adaptor that includes a poly(T) at its 3'-end and a UPL binding region. c) Amplification of the cDNA library with a miRNA-specific forward primer and a generic reverse primer complementary to the universal, adaptor and quantified using UPL probe.
Using this method we could detect various hsa-miRNAs in several cell lines with very high sensitivity, detecting as little as 3.8fM of spiked RNA. The assay could also discriminate miRNAs that differ by as little as a single nucleotide, exhibiting high specificity in addition to high sensitivity.

Session Single-cell qPCR
Chair B. Liss & A. Stahlberg
Lecture hall HS 15

Session sponsored by:

RT-qPCR of individual dopamine neurons form mouse brains and human post mortem brain sections.
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The dopamine midbrain system and the activity of dopamine releasing (DA) midbrain neurons is not only involved in motor control and movement disorders like Parkinson disease but also plays a crucial role in emotional and cognitive brain functions, and related disorders like schizophrenia, drug addiction, and attention-deficit-hyperactivity-disorder.
Our main research goal is to define the functional and molecular mechanisms of different types of DA midbrain neurons, which control their distinct physiological roles and their selective transitions to disease states (Liss&Roeper, Brain Res. Rev., 2008). By combining in vivo retrograde tracing with in vitro brain slice electroyphysiology and UV-laser-microdissection, as well as with quantitative RT-PCR based gene-expression profiling at the single cell level (Liss&Roeper, TINS 2004; Gründemann et al, NAR, 2008), we aim to define the pathophysiological signalling-pathways that control DA neuron activity as well as activation and execution of selective disease pathways of the dopamine system, in particular in Parkinson's disease.
We focus on the role of ion-channels and receptors as their cell-specific activity directly defines neuronal activity in health and disease states (Lammel et al. Neuron 2008). To address these issues, we analyze cellular function as well as mRNA-expression of individual DA neurons from control mice and respective disease mouse-models as well as from post mortem human brain-tissue. To increase specificity, we developed a cell-specific approach for RT-qPCR that also takes the important issue of variable RNA integrities human post mortem brain samples into account.

Technical aspects of mRNA quantification in single cells using RT-qPCR
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Gene expression has a strong stochastic element resulting in highly variable mRNA levels among individual cells, even in a seemingly homogeneous cell population. Access to fundamental information about cellular mechanism, such as correlated gene expression, motivates studies of multiple genes expressions in individual cells. Quantitative reverse transcription PCR (RT-qPCR) is the most versatile method that provides sufficiently accurate measurements of mRNA levels in single cells. Technical considerations related to reproducible and efficient sampling, lysis, reverse transcription and real-time PCR will be presented, and we will show that technical noise in single cell RT-qPCR is insignificant compared to biological cell-to-cell variations in mRNA levels for medium and high abundant transcripts. Data from human embryonic stem cells, mouse β-cells and primary astrocytes will be presented and used to demonstrate various aspects of gene expression profiling in individual cells.

AmpliGrid and AmpliHyb, a new miniaturized, multiplex qPCR system for single cell analysis
Gordana Cerovic[2], Marianna Alunni[1], Angelique le Bras[2], Régis Melizzi[2], Jean-Luc Grabias[2], Maxime Rattier[2], Martin Kantlehner[1], Petra Hartmann[1], Wolfgang Mann[1] and Claude Weisbuch[2]
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Many basic questions in Biology and Medicine demand methods for the analysis of the basic unit of life, the single cell.
We developed AmpliHyb, an innovative real-time PCR instrument which allows the efficient quadruplex qPCR and qRT-PCR reaction in only 1 µl. The AmpliHyb is a small footprint instrument integrating the PCR-cycler and fluorescence reader, which uses AmpliGrid slide as a consumable (Olympus). AmpliGrid is a chemically structured microscope slide for performing PCR reactions within droplets having a volume of only 1µl each. Up to 48 PCR reactions with a total volume of only 48µl (48x1µl; each reaction centre has 1.6mm diameter) can be realized on an AmpliGrid. The AmpliGrid slide is transferred into the AmpliHyb which runs direct amplification, detection and analysis of single cells in real time mode. The result file contains quantitative PCR data on the single cell analyzed (qPCR).

Part of the system is a special SlideCycler developed by Olympus based on a resistive heater and a fan for cooling. This combination is taking advantage of the AmpliGrid's unique thermal properties (low thermal mass and good heat conductivity). The readout is performed with the fluorescence imaging system developed by Genewave. We present here the results obtained on the Ampligrid slide combined with the AmpliHyb instrument, using different experimental model systems. Most notably, results confirmed that the AmpliHyb system combined with the AmpliGrid chip is able to detect single cell as template by means of a multiplex qPCR approach.

Post-characterization of cultured pituitary cells using single-cell real-time RT-PCR

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As part of our investigations of the gonadotropin-releasing hormone (GnRH) systems in Atlantic cod, we have developed a single-cell real-time RT-PCR assay to phenotype pituitary gonadotrope cells following patch-clamp registrations.
The assay is based on SYBR green I detection, and has been optimized using specific primers designed from cod gonadotropin subunit (FSHβ and LHβ) mRNAs. EF1a was used as a qualitative reference control for all cells tested. By having one of the primers in each pair spanning an exon-exon border, and after careful testing on genomic DNA, we could omit the time-consuming and potentially RNA degrading DNase treatment. High PCR specificity and efficiency was achieved only using primer pairs defining relatively short PCR products of about 60 to 80 bp. During single cell handling, the cell content was transferred to a sodium citrate buffer (RNA storage solution), before frozen on liquid nitrogen and stored at -80 °C until cDNA synthesis.
In present study we also developed a single-cell real-time RT-PCR assay using a rat pituitary cell line (GH4) and with primers specific for prolactin mRNA. This assay was developed for comparing whole cell harvesting with harvesting of cell contents through a patch pipette, using both the GH4 cell line and primary dispersed cells from cod pituitaries. The results revealed interesting differences regarding extracellular mRNA contamination.
Whole cell harvesting followed by cell lysis and real-time RT-PCR worked well on the GH4 cells. However, harvesting of whole cells from the cod primary pituitary cultures regularly produced false positives. We believe that the false positives result from RNA leakage from ruptured cells, caused by the longer trypsin exposure and the rough mechanical dissociation needed to disperse cod pituitary cells. This is in contrast to the gentle treatment necessary to maintain a stable cell line such as the GH4. In addition, our experiments show that RNA in the extracellular solution had a tendency to stick to untreated patch pipette glass, probably through charged residues. This source of contamination can be eliminated by silanizing the glass using Sigmacote, a chlorinated organopolysiloxane in heptane. However, we experienced a reduced chance of obtaining giga seal using Sigmacote, thereby making the patch-clamp experiments difficult to perform. By diluting the Sigmacote 1/15 with heptane, the conditions for forming giga seal was significantly improved, while still excluding false positives in the single-cell real-time RT-PCR assay.
We conclude that harvesting of cytosol using patch pipettes is the preferred method for collecting mRNA from cod primary pituitary cultures, and that silanizing the glass is necessary to avoid mRNA contamination. Furthermore, single-cell real-time RT-PCR assay is a feasible and promising method for post-characterization of individual cells in culture.

Molecular characterization of circulating tumor cells in large quantities of contaminating leukocytes by a multiplex real-time PCR

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Circulating cells with the characteristics of tumor cells of epithelial origin have been demonstrated in blood and bone marrow of prostate, melanoma, colon, esophageal, head and neck, lung and breast cancer patients. These cells have not only been shown in patients with metastatic disease, but also in those whose tumors are apparently localized. The development and optimization of new technologies to identify and characterize such cells, and the establishment of the association of their presence with potentially clinical significance are highly relevant. A recently developed technology to quantify the number of CTCs in whole blood of cancer patients is the use of the CellSearch™ CTC Test (Veridex LLC, Warren, NJ), so far the only US Food and Drug Administration (FDA) approved diagnostic test to automate the detection and enumeration of CTCs for monitoring disease progression and therapy efficacy in metastatic prostate, colorectal and breast cancer. In addition to enumeration, there is great interest in molecular characterization of CTCs to understand fundamental issues such as their genetic composition, and to establish association of these genetic profiles with patient treatment outcomes. However, although this system allows capture of CTCs in blood of cancer patients, there are still considerable quantities of contaminating leukocytes present after enrichment. This contamination, together with the fact that CTCs detected by CellSearch™ CTC Test appear to occur in small numbers in humans (frequently fewer than 5 CTCs per 7.5 mL blood in metastatic breast cancer patients), forms a pitfall that can not be ignored when one is interested in a molecular characterization method specific for CTCs. The purpose of this study was to optimize a robust method to perform mRNA expression analysis of up to 96 genes by real-time RT-PCR, thus conveniently fitting a 96-well plate, on as little as 1 CTC in an environment containing large quantities of contaminating leukocytes. By using a set of genes with no or minor expression by leukocytes, we succeeded to perform quantitative gene expression profiling specific for as little as one breast cancer CTC present in a CTC-enriched environment typically containing over 800 contaminating leukocytes. In summary, our study shows that it is possible to perform mRNA expression analysis of up to 96 genes specific for as little as one cell. With the restriction of not using genes more dominantly expressed by CellSearch-enriched leukocytes, any gene set specific for any cancer type can be implemented in the method we describe. The resulting data can be used to further characterize cancer type specific CTCs, thereby potentially improving our insight into biological processes and ultimately patient management.

Visualization of Single mRNA Molecules

Fay Wang[1], John Flanagan[1], Yunqing Ma[2], Steve Lai[2], Takuro Yaoi[2], Son Bui[2], Li-chong Wang[1], Jennifer Wong[1], Nan Su[1], Jessie Wu[2], Nina Nguyen[2], Aiguo Zhang[2], Steve Chen[1], Frank Witney[2], Quan Nguyen[2] and Yuling Luo[1]
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The majority of mRNA transcripts of functionally relevant genes are present at fewer than 10 copies per cell, which cannot be detected by current in situ hybridization technologies. We report here the development of a highly sensitive and specific in situ hybridization technology which enables robust and cost effective detection of native mRNA transcripts in single cells. The assay utilizes a simultaneous signal amplification and nonspecific hybridization suppression strategy to achieve a very high signal-to-background ratio, sufficient for single copy mRNA detection under a standard fluorescent or bright field microscope. In addition, the fluorescent-based assay enables multiplex detection of RNA transcripts allowing for comparative analysis of single cell gene expression profiles in large cell populations. The technology has been used for robust detection of a variety of low expression genes in cell lines, PBMCs, and FFPE tissue sections, and has been adopted for automation and high throughput analysis. This technology thus provides the first opportunity to see the innerworkings of individual cells at the transcription level for virtually every gene in the human genome.

Session **Tutorials in qPCR BioStatistics & BioInformatics**
Chair **J. Vandesompele & A. Forootan**
Lecture hall **HS 15**

TUTORIAL: Easy analysis of qPCR data with state of the art quantification models and comprehensive quality controls using qBasePlus.
Jan Hellemans[1,2], Stefaan Derveaux[1] and Jo Vandesompele[1,2]
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Quantitative reverse transcription PCR (RT-qPCR) is considered today as the gold standard for accurate, sensitive and fast measurement of gene expression. Successful expression analysis not only requires proper experiment design and careful reaction setup and monitoring, but also depends heavily on appropriate calculations and data analysis. Biogazelle's qBasePlus software enables easy, fast and realible processing of qPCR data with state of the art quantification models and comprehensive quality controls (http://www.qbaseplus.com) qBasePlus is built upon the proven Ghent University geNorm and qBase technologies (with more than 1500 citations in PubMed), delivers correct quantification with efficiency correction and multiple reference gene normalization, and provides tools to correct for (often underestimated) technical run-to-run variation. To guarantee reliable results, quality controls are implemented wherever possible, including tests for replicate variability, control sample results and reference gene stability. In qBasePlus advanced quantification models and quality controls go hand in hand with its ease of use. A large numbers of importers are available to allow easy data importfrom almost all qPCR instruments available, and data or results can be exported for exchange with colleagues or for publication in an RDML compliant format. Last but not least, qBasePlus includes a data management system to keep track of all performed runs and experiments.
In this tutorial we will demonstrate how a 4-run experiment can be fully processed in just 24 seconds. We will also show how easy it is to analyze large TaqMan array experiments and how quality controls help you to evaluate your experimental data.

TUTORIAL: RefGenes - a new tool to find suitable reference genes for selected experimental conditions
Philip Zimmermann
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Reference genes are frequently used as internal controls to normalize qRT-PCR experimental data. Classical genes such as GAPDH or ACTB are commonly used, based on the a priori assumption that their expression level is stable. Although they seem to be stable under certain conditions, in several reported cases they have been shown to be unsuitable for normalization because their expression varied significantly in the tested conditions. Ideally, one would want to know in advance which genes are most stable for one's own experimental context, and to be able to choose from a genome-wide set of candidates. RefGenes is a new tool that aims at finding the most suitable reference genes for a chosen set of conditions. Because it makes use of Genevestigator's extensive collection of curated experiments (>25,000 Affymetrix arrays), the user can choose from a broad choice of conditions those that are similar to his own experiment (similar tissues, or similar treatments), and find which genes were the most stable across all microarrays associated with these conditions. Validation experiments have shown that, in the conditions tested, the new reference genes proposed by RefGenes outperformed all commonly used references genes. RefGenes is easy to use and freely available for academic users. Reference genes can be searched for human, mouse, rat, Arabidopsis, rice, barley, soybean, and soon also microorganisms.

TUTORIAL: Data analysis for gene quantification and expression profiling using GenEx.
Anders Bergkvist
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As the data size and complexity from qPCR projects increase, the need for comprehensive automated or semi-automated software tools increase rapidly. Software tools can provide support after data collection by providing data pre-processing, statistical analysis and visualization capabilities, often for hypothesis generating purposes. Alternatively they can be used prior to experimental realization by helping to define experimental design parameters for hypothesis validation assays. The GenEx software from MultiD Analyses AB provides all of the capabilities mentioned above. Performing accurate qPCR data pre-processing is very important, particularly for quantification purposes. Many steps are usually implemented and it is useful to follow protocols in order to avoid introduction of unwarranted processing variability and bias. The protocol available in GenEx is easy to adapt to user-specific needs while at the same time comprehensive enough to enable users to easily perform accurate pre-processing. As scientists we believe observations we make are manifestations of rational processes. Our challenge is to identify the particular rational process that we want to study while minimizing contributions from rational processes that would obscure the understanding of our particular study. Contributions from unwanted processes are often called random although the underlying processes may not be. Tools to differentiate between contributions from desired and unwanted processes include parametric and non-parametric statistical tests, scatterplots, principle component analyses and neural network analyses. These tests and analyses and more are available in GenEx. Validation of scientific conclusions is not absolute, but based on reproducibility. No scientific theory is above scrutiny and potential revision. However, based on certain assumptions, an increasing number of observations that supports a particular conclusion will also increase our confidence that the particular conclusion is going to continue to be supported by future observations. We may thus define a level of confidence by which we would assign our conclusion to be “true”. A good experimental design aimed to validate a hypothesis should therefore include the number of necessary observations needed to obtain the desired level of confidence, before realization of the experiment. Based on a hypothesis of an observed effect, including desired level of confidence, amplitude of desired observed effect and variability of confounding effects, GenEx can calculate the number of necessary observations. The presentation will focus on important considerations for running qPCR experiments and ways the GenEx software may provide support.

TUTORIAL: CAmpER - An open analysis framework for real-time PCR data using single sample amplification efficiency calculation.
Jochen Blom[1], Lukas Jelonek[1], Jörn Kalinowski[2], Christian Rückert[2] and Alexander Goesmann[1]
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Quantitative real-time PCR has become one of the fundamental methods of modern molecular biology and medicine for the measurement of gene expression. For an exact analysis of the results of a real-time PCR experiment it is crucial to have exact knowledge of the amplification efficiency of each single sample. Therefore the software CAmpER was designed as a web based application for the automatic analysis, annotation, and storage of real-time PCR experiments performed with different real-time PCR systems. CAmpER currently supports four different real-time PCR platforms: Lightcycler2 and Lightcycler480, Opticon and Rotorgene 3000. Fluorescence and melting curve data is automatically imported either from the raw output files or from TAB-separated data exported by the software of the corresponding system. The amplification independent background fluorescence is eliminated by fitting a saturation function to the data points of the early linear phase and subtracting this saturation function from the fluorescence curve [1]. Based on this normalized fluorescence data the amplification efficiency is calculated for every single sample by two different independent algorithms: The DART-PCR method [2] and the four parametric logistic model (FPLM)[3]. In the DART method the amplification efficiency is calculated by computing the slope of the log-linear phase of the fluorescence curve. The FPLM method calculates the efficiency by estimating the exponential phase of the fluorescence curve and fitting an exponential function to it. The algorithms used for the calculation of amplification efficiencies were verified by wet lab experiments. The calculated amplification efficiencies are used to calculate efficiency-corrected crossing points. These corrected crossing points allow us to analyze relative gene expression of samples quite more accurately in comparison with the calculation of gene-expression with uncorrected crossing points. CAmpER allows users to join samples from multiple PCR runs to specify biological and technical replicate groups. Gene expression ratios can be calculated for these replicate groups using the ΔΔCT method. The

significance of the results is evaluated by a Fisher-Pitman permutation test. CAmpER provides basic LIMS functionality by integrating the annotation of both RNA sample and experimental setup with consistent storage of all relevant information of a real-time PCR experiment in a relational database system. To guarantee easy access to stored data and easy use of the implemented functions a concise user interface for CAmpER was designed. This user interface is implemented as a web-based front-end, making a local installation of CAmpER needless. By now CAmpER has more than 60 registered users. The service is available at the following URL: http://camper.cebitec.uni-bielefeld.de

[1] Wilhelm *et al.* : BioTechniques, 34(2), 2003
[2] Peirson *et al.* : NAR, 31(14): e73, 2003
[3] Tichopad *et al.* : NAR, 31(20): e122, 2003

Wednesday 11th March 2009

Session High-Throughout session
Chair J. Hellemans & K. Livak
Lecture hall HS 14

Session sponsored by:

Moving from qPCR Assays to qPCR Arrays

Kenneth James Livak
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The paradigm for gene expression analysis has been the use of microarrays on a few samples to survey a large number of genes, followed by the use of individual qPCR assays on a much larger number of samples to validate the candidate genes identified in the microarray screen. Advances in microfluidics have dramatically increased reaction densities so that thousands of individual assays can be performed on a single qPCR array. This means it is now possible to consider using qPCR for the survey step in gene expression analysis, especially on limited samples such as single cells. In order to maximize the number of genes that may be analyzed in parallel on a Fluidigm dynamic array, a novel method has been developed that involves combinatorial mixing of forward and reverse primers to generate functional qPCR assays. This approach has been used to create qPCR arrays that interrogate 1152 genes on four samples with duplicate assays.

Accurate and Objective Gene Copy Number Profiling using Real-Time PCR

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Real-time quantitative PCR (qPCR) is the gold standard for accurate quantification of nucleic acids. While it is widely applied for gene expression analysis, the method is also perfectly suited for detection of copy number variants. We have previously reported that multiple technical replicates, reference targets and inter-run calibrators improve the accuracy of the results (Hellemans et al., Genome Biology, 2007). Here, we extend this series of 'more is better' to include multiple reference samples for more accurate determination of copy numbers, whereby the inclusion of both a normal and a deletion control as a reference was shown to result in less false positive copy number variants. We further outline a data analysis strategy based on z-score distribution of copy numbers in normal controls for statistical assessment of copy number variation in patients.
This approach was first evaluated in a small scale screening for exon deletions in the *LEMD3* gene of 37 mutation negative osteopoikilosis or melorheostosis patients. Twenty-two normal samples were used to determine assay performance and normal variation in measured copy numbers. In this experiment, the outlined quantification strategy resulted in more accurate and objective copy number profiling. Consecutively, a large scale screening for copy number variants was performed in the *SHOX* and *ABCA4* gene for 95 patients with Leri-Weill dyschondrosteosis or idiopathic short stature and 60 patients with Stargardt disease, respectively. For this study, including the assessment of assay variation in 30 normal controls, 14,544 qPCR reactions were performed in thirty-nine 384-well plates. Low volume (8 µl) reactions were used to reduce the cost, and the qBasePlus real-time PCR data-analysis software (http://www.qbase_plus_.com) with export to custom Excel sheets for downstream processing were applied to streamline gene copy number analysis and interpretation. This experiment demonstrated that qPCR is perfectly suited for routine diagnostic copy number variation analysis at low cost and short turnover times.

High-Throughput Analysis of Nucleic Acids Using the LightCycler® 1536 qPCR Platform

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An important trend in biological and biochemical analysis over the past 15 years has been miniaturisation and parallelisation of analytical procedures. Large-scale high-throughput analysis of gene expression is an essential element of modern functional genomics where individual samples are screened against many thousands of target genes. Initial expression analysis is mainly based on DNA microarrays due to their highly parallel readout and throughput capability. According to its superior sensitivity and accuracy, qRT-PCR is the well-established gold standard for precise profiling of gene expression levels of selected gene patterns. Scaling qPCR to a higher throughput with concurrent miniaturisation of individual reactions provides a new generation of enabling nucleic acids analysis systems combining the strengths of qPCR with the parallelism and throughput capability of low-density microarrays. The LightCycler® 1536 is a novel system capable of performing qRT-PCR-based DNA/RNA analysis in an array-like format at high-speed in a proprietary multiwell 1536 plate. Here we present concrete examples of qRT-PCR applications using the LightCycler® 1536 system demonstrating analytical performance, robustness and flexibility regarding different kinds of PCR set-up workflows.

Measurement of Gene Expression by Massively Parallel Nanoliter real-time PCR

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The complexity and subtlety of discoveries in genetic variation continues to arise a pace. Current tools are inappropriate or too costly to validate discoveries, to make correlations and to test hypothesis. The BioTrove OpenArrayTM NT Cycler system extends the reach of genomic discovery, validation and quantification processes by means of a unique parallel array format. Using proven solution-based qPCR chemistries, the system delivers outstanding analytical performance with medium throughput, with an ideal sample-feature ratio. The novel through-hole OpenArray,TM device facilitates the easy transference of many qPCR applications and assays to the new technology platform. The system integrates to standard sample prep/workflows. The novel miniaturization device lowers the reaction volume overcoming the problems with of low-volume sample evaporation, or sample absorption, while delivering lower cost per sample. Up to 3072 individual solution-phase reactions are run in parallel, in 33nl though-holes on the size of a microscope slide in a software-controlled, standardized of a thermal cycler. The User is thus empowered to extend and test discoveries across more samples in applications in genotyping, endpoint, real-time and DNA methylation analysis. My presentation will describe the Biotrove OpenArray TM NT Cycler system, how the system meets the challenge of delivering more while lowering costs. Helping drive discoveries in gene signatures, quantitative trait loci, bridging the gaps in validation in toxicogenomics and pharmacogenomics towards translational medicine. Perhaps helping the development of better, more informed and cost effective diagnostic/treatment platforms.

QuantPrime – a flexible tool for reliable high-throughput primer design for quantitative PCR

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Medium- to large-scale expression profiling using quantitative polymerase chain reaction (qPCR) assays are becoming increasingly important in genomics research. A major bottleneck in experiment preparation is the design of specific primer pairs, where researchers have to make several informed choices, often outside their area of expertise. Using currently available primer design tools, several interactive decisions have to be made, resulting in lengthy design processes with varying qualities of the assays. Here we present QuantPrime, an intuitive and user-friendly, fully automated tool for primer pair design in small- to large-scale qPCR analyses. QuantPrime can be used online through the internet (http://www.quantprime.de/) or on a local computer after download; it offers design and specificity checking with highly customizable parameters and is ready to use with many publicly available transcriptomes of important higher eukaryotic model organisms and plant crops (currently 295 species in total), while benefiting from exon-intron border and alternative splice variant information in available genome annotations. Experimental results with the model plant Arabidopsis thaliana, the crop Hordeum vulgare and the model green alga Chlamydomonas reinhardtii show success rates of designed primer pairs exceeding 96 %. QuantPrime constitutes a flexible, fully automated web application for reliable primer design for use in larger qPCR experiments, as proven by experimental data. The flexible framework is also open for simple use in other quantification applications, such as hydrolyzation probe design for qPCR and oligonucleotide probe design for quantitative in situ hybridization. Future suggestions made by users can be easily implemented, thus allowing QuantPrime to be developed into a broad-range platform for the design of expression assays.

Oligo Design Across the Mouse Genome

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Fluorescence-quenched probes are routinely used to gauge gene copy number. We describe a bioinformatic engine for the design of such oligos, and used to generate five thousand TaqMan assays for the NIH Knockout Mouse Project (KOMP). Analysis of this data-set uncovers important trends in amplification performance. A demonstration of this publicly-available software program highlights those considerations that become pronounced in large-volume sequence design, including specificity, redundancy and accessibility. Based on this experience as well as user feedback, new software functionality is introduced to improve upon the original sequence designs.

A novel digital technology for non-enzymatic direct multiplexed measurement of gene expression

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We describe a novel technology, the nCounter system, for highly multiplexed analysis of gene expression levels. Then nCounter system detects individual mRNA molecules using an assigned code sequence of fluorescent molecules, and counts the number of times that code appears in a sample. No enzymes are used in our system; rather, the collection of probes is hybridized in solution to RNA in a sample. Experiments performed in a single multiplex analysis of 550 human genes revealed a correlation coefficient of 0.999 between replicate measurements, a detection limit between 0.1fM (0.2 copies/cell) and 0.5fM (1 copy/cell), and a linear 500-fold dynamic range. The nCounter system can detect a 1.5-fold increase or decrease in expression across a broad range of expression, and as little as 20% changes in expression for genes present between 1fM and 10fM. We demonstrate a good correlation between nCounter system and Affymetrix GeneChip technology, and better correlation with TaqMan, for –fold change measurements using two different experimental paradigms. Furthermore, a comparison of transcript levels measured by the nCounter system with SYBR green RT-PCR demonstrated a high correlation in the gene expression pattern at all transcript levels. We show that a whole cell lysate can be used as starting material with equivalent results to purified total RNA. Finally, we show that RNA extracted from formalin-fixed paraffin embedded (FFPE) tissues can be used in the nCounter system to analyze expression levels in archived samples. Our unique direct detection and digital quantification approach results in unprecedented sensitivity, precision and reproducibility in gene expression analyses.
Materials & Methods - nCounter hybridization reactions were performed in triplicate with total RNA samples isolated from mock and polio virus infected human A549 cells. nCounter reactions were set up as follows: 100ng of total RNA - Reporter and capture probes for 509 human mRNAs and controls made to non-human sequences (6 positive, 2 negative) DNA control targets spiked in at 0.1, 0.5, 1, 5, 10 and 50 fM Hybridizations were carried out for 20h at 65°C. Excess reporters were then removed by using magnetic bead based purification. The same samples and amount of RNA were also analyzed with Affymetrix® U133Plus2 arrays, using the two-cycle amplification/labeling protocol recommended by the manufacturer. We selected a subset of 14 genes in which the measured log2 fold-change was significant in one platform but not the other for further analysis by TaqMan Real-Time PCR. In a second experiment, nCounter hybridization reactions were performed in triplicate as described above with total RNA samples isolated from sea urchin embryos collected at seven different development time points. A set of 21 genes were selected for comparison with existing SYBR Green Real-Time PCR data generated in the Davidson Lab.

Sensitive and high throughput multiplexed immunoassays for biomarker discovery in biobanked samples using proximity ligation assays and qPCR.

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Methods consuming small sample amounts and allow for sensitive screening of large numbers of putative biomarkers indicative of disease are required to advance biomarker discovery and validation. We present multiplex proximity ligation assays (PLA) were protein analytes are converted to unique DNA amplicons and subsequently detected by high throughput quantitative PCR. These multiplexed profiling panels solve many analytical problems found in biomarker verification, such as sensitivity (low femto Molar), multiplexing, throughput, and sample consumption (one micro Litre of plasma). As the assay can use either matched monoclonal antibody pairs or even single batches of polyclonal antibodies both with high detection specificity, a wider range of potential biomarkers can be targeted compared to conventional immunoassay methods. In another incarnation of PLA performed in situ, the assay can precisely quantify proteins and protein interactions in FFPE tissue providing localized data through subjective single molecule counting and fluorescence microscopy. The ability to quantify protein modifications and protein-protein interactions in situ using pairs of primary target specific antibodies opens a new realm of biomarker opportunities particularly with in drug response markers. Olink Bioscience in Uppsala is coordinating a EU-funded effort to expand our capabilities to profile hundreds of putative plasma biomarkers in minute amounts of biobanked samples in a project called PROACTIVE. A high throughput and multiplexed platform for biomarker research will be built based on the proximity ligation assay (PLA). Proof of principle data has recently been published for multiplexed PLA as a profiling tool for low abundance cancer biomarkers. The enabling power of this technology will make this project comprise the largest immunoassay based effort to find novel biomarkers for colorectal cancer ever performed in a single sample collection. Once in place, the technology platform will become a valuable resource for the diagnostics and pharmaceutical communities in need of capable tools to find novel biomarkers and biomarker panels.

Session qPCR BioStatistics & BioInformatics
Chair M. Kubista & A. Tichopad
Lecture hall HS 14

Real-time PCR Expression Profiling - Concept of multiway profiling

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The large sensitivity, high reproducibility and essentially unlimited dynamic range of real-time PCR to measure gene expression in complex samples provides the opportunity for powerful multivariate and multiway studies of biological phenomena. In multiway studies samples are characterized by their expression profiles to monitor changes over time, effect of treatment, drug dosage and so forth. Here we perform a multiway study of the temporal response of four yeast Saccharomyces cerevisiae strains with different glucose uptake rates upon altered metabolic conditions. We measured the expression of 18 genes as function of time after addition of glucose to four strains of yeast grown in ethanol. The data were analyzed by matrix-augmented PCA, which is a generalization of PCA for 3-way data, and the results are confirmed by hierarchical clustering and clustering by Kohonen self-organizing map using the GenEx software (www.multid.se). Our approach identifies gene groups that respond similarly to the change of nutrient, and genes that behave differently in mutant strains. Of particular interest is our finding that ADH4 and ADH6 show a behavior typical of glucose-induced genes, while ADH3 and ADH5 are repressed after glucose addition. We conclude multiway real-time PCR gene expression profiling is a powerful new technique that can be utilized to characterize functions of new genes by, for example, comparing their temporal response after perturbation in different genetic variants of the studied subject. The technique also identifies genes that show perturbed expression in specific strains.

Design and analysis of Q-RT-PCR assays for haematological malignancies using mixed effects models

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The recent WHO classification of haematological malignancies includes detection of recurrent genetic abnormalities with prognostic significance [1]. Consequently, an increasing number of specific real-time quantitative reverse transcription polymerase chain reaction (Q-RT-PCR) based assays are in clinical research use and needs quality control for accuracy and reproducibility.
The standard analytical technique is to use the Delta-Delta-Ct method. Although this method accounts for sample specific variations such as RNA purification, it does not account for other experimental effects like variations in amplification efficiency and assay variation. A factorial design allows the effect of several factors and even interactions between them to be determined.
To obtain an assessment of the accuracy and precision of the assays a novel approach for the statistical analysis of Q-RT-PCR has been developed based on mixed effects modelling of factorial designs. This approach extends the recently suggested use of analysis of variance for Q-RT-PCR experiments [2,3,4,5]. A new stochastic model for the fluorescence measurements has been introduced, which accounts for varying efficiency, inhomogeneous variance, repeated measures correlation and experimental variations. This leads to improved accuracy and precision of quantification of gene expressions. Especially, the graphical display facilities were useful during the various phases of the process, starting with exploratory plots of the data and concluding with diagnostics plots in order to assess the adequacy of the fitted model.
The modelling approach has been used to conduct fold change analysis on data concerning RNA in arabidopsis thaliana from [5] and data from our own lab regarding microRNA expressions in Diffuse Large B-cell Lymphoma. The analysis was implemented in the freely available software package *R* . In general *R* and its contributed package Bioconductor is gaining more and more importance in gene expression analysis and it is therefore a natural choice for the analysis of high throughput Q-RT-PCR experiments.

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Adequate experiment design as the first important step in obtaining valid biological inference with qPCR technique

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Experimental design is a structured, organised method that is used to determine the relationship between the different factors affecting an experimental process and the output of that process, first developed by Sir Ronald A. Fisher; the renowned mathematician and geneticist. Employing quantitative PCR (qPCR) to study gene expression [2,3,4] requires statistical considerations of all invoked factors: the treatment effect, the inter-subject biological variance, and the noise introduced due to sample processing. In addition, the gene-specific effect on the error structure must be considered. Typically, an experiment testing a hypothesis stating a difference due to an experimental factor comprises steps of composing treatment groups of biological subjects, sampling biological material, extracting the RNA, reverse transcription (RT) of the RNA into cDNA, and amplifying the cDNA by the qPCR. We show how various sample processing steps affect the assay when working with solid tissue, blood, cell culture or single cell. We further propose model for making inference about biological effect that takes into account noise propagation throughout various sample processing step. Eventually, software tool utilising intuitive graphical user interface was designed to facilitate experiment planning in terms of adequate replicate number and location, easy grouping of data into replicate groups, quality control and statistical testing.

Modeling Real-Time PCR Efficiency and Fluorescent Signal intensity for accurate gene quantification using a single standard

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Objectives : Several methods are available to quantify a viral load in a sample subjected to a real-time PCR reaction. Nevertheless the construction of a standard curve and a hypothesis on the amplification efficiency are still required. We propose an innovative mathematical method , based on a model of the amplification rate of the PCR reaction. This technique estimates the initial fluorescence value of a sample, which is theoretically proportional to its DNA content, making thus possible the absolute quantification of a given sample with a single standard .
Methodology : Hepatitis B Virus (HBV) DNA was extracted from a single positive control containing 107 copies/ml. A serial dilution of this sample was done to obtain five aliquots containing 50 000, 5 000, 500, 50 and 10 copies/PCR. 12 replicates of each concentration mix were distributed on a raw of a 96-wells plate. The Q-PCR data acquisition was performed on a Chromo4 apparatus (Bio-Rad, Hercules, CA, USA).
For each curve, a specific background adjustment was applied in this way : i) identification of the Cmin cycle that corresponded to the end of the background phase of the curve, and ii) removal of the estimated background trend, either linear or nonlinear, within the interval from cycle 1 to Cmin from the signal.
Results : The best fit of the efficiency of the amplification was obtained by a model dived into a constant phase and an exponential phase (figure B). An estimation of the initial fluorescence value F0 was deduced from the transposition of the efficiency model on the amplification curve (figure A).
The plot of log10(F0) as a function of log10(N0), N0 being the initial concentration of the sample, gave a straight line similar to a

standard curve. To rank our method with the traditional Ct and Second Derivative Maximum methods, we then compared their ability to quantify unknown samples using a standard curve. At each concentration, simulations showed that the three methods gave comparable results both for the predicted target value and for the standard error. According to the proportionality between F0 and N0, we finally tested our method with a single standard point. Whatever the standard concentration used, the means of the estimated quantities were still very close to the expected values and comparable to those obtained with the other methods. The maximal error was of a factor of 2 on the mean, and a factor of about 3 on a single point. Conclusion: We present a new method to analyze Q-PCR data which is fully automatized and requires only one standard point for precise and reliable target determination. One advantage of our method is that it gives an independent estimate of the amplification efficiency of each sample, useful to detect a potential inhibition of the reaction. Finally, it could help to reduce times and costs for clinical applications.

The Delta-TF Method for Real-Time PCR Data Standardization

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Initial (unprocessed) curves of DNA accumulation obtained with the use of real-time PCR Cyclers contain a number of external (not related to the process studied, nonbiological) artifacts that distort the results of experiment. The shape of the curves is influenced by errors of preparation of the reactions, dispersion of optical characteristics of the tubes, heterogeneities in the optical scheme of detecting amplifiers, noises of the electronics, etc. To decrease the effect of these factors on the results of the study, modern algorithms of analysis of the curves of DNA accumulation include a stage of data normalization. In this work, we propose an effective method for exclusion of the effect of nonbiological factors on the resulting curves of DNA accumulation during real-time PCR, delta-TF method. The method is based on recording the fluorescence of a reaction mixture that contains a fluorescent substance with a strong dependence of fluorescence intensity on temperature. Recording is performed at two different temperatures during the first several cycles of PCR followed by calculation of the normalizing coefficients on the basis of the measured difference in the fluorescence intensity.

Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data.

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Nowadays, real-time RT-PCR (qPCR for short) is a well established technique. Nonetheless, comparison of qPCR data analysis methods reveal considerable differences in variability and bias, which in turn may lead to loss of sensitivity and, more importantly, opposing biological conclusions. In qPCR, the increase in amplicon concentration per cycle is given by Nc=N0.E^C, in which N0 and Nc are the amplicon concentrations, expressed in arbitrary fluorescence units, at the start and after C cycles and E is the amplification efficiency. The number of cycles (Ct) needed to reach a fluorescence threshold (Nt) can then be used to calculate the starting concentration (expressed in fluorescence units) with N0 = Nt/E^Ct. Most current analysis methods are based on re-arrangements of this basic equation and thus depend on the proper estimation of the PCR efficiency. Different efficiency values are used in this equation and have been shown to lead to differences in variation and bias. We now report that a significant part of this variation can be traced back to errors in baseline correction. A baseline estimation error of just 1% leads to a deviation in the efficiency of about 1%, and, at a Ct of about 25 cycles to a variation in starting concentration of around 30%. Surprisingly, baseline correction is hardly being discussed in papers on qPCR analysis and is generally delegated to undisclosed algorithms in the software that came with the PCR apparatus. The baseline depends, among others, on cDNA and primer concentrations, primer choice and unbound fluorochrome. However, because the nature of the interactions between those variables is unknown, the observed fluorescence in the first cycles is unsuitable for baseline determination. The observed fluorescence can be considered to reflect the sum of the baseline and an exponential increasing amplicon concentration until the efficiency plummets and the plateau phase is reached. Based on the constant efficiency during the exponential phase cycles, we implemented an iterative search for the baseline value that results in the longest straight line of data points downwards from the plateau phase when plotted on a semi-logarithmic scale. This procedure isolates the amplicon dependent part of the observed fluorescence values. The PCR efficiencies and Ct values in the resulting baseline-corrected samples can be determined. The variation in the resulting efficiency values and starting concentrations is significantly reduced, and the expression ratio is unbiased when the mean of the PCR efficiencies of the samples per amplicon is used in the calculation. It has long been shown that bias in qPCR observations originates from ignoring the difference in PCR efficiency between amplicons. Detection of such a difference in efficiencies requires that the fluorescence baseline is properly corrected in all individual PCR amplification curves. The LinRegPCR data analysis program was updated to perform baseline estimations per sample.

The calculation of real-time PCR ratios by means of Monte Carlo Simulation or high-order Taylor expansion.

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A crucial point in the quantitative analysis of real-time PCR is the question of how the error of efficiency estimation and threshold cycle calculation is propagated to the final calculation of ratios between samples. Several methods that address this problem have recently been discussed, which are the following:
1) Using the first-order Taylor expansion on the errors estimated from threshold cycles and the efficiency obtained from a dilution calibration curve (Karlen et al., BMC Bioinformatics 2007, 8:131)
2) An approach similar to 1) by using Gaussian error propagation as implemented in the qBase quantification framework (Hellemans et al., Genome Biology 2007, 8:R19)
3) Using a permutation regime by reallocating the threshold cycles between sample and control groups as implemented in the different REST software versions (Pfaffl et al., NAR 2002, 30:E36)
4) An extended Gaussian error propagation approach that also takes the covariance structure between the estimated variables into account (Nordgard et al, Anal Biochem 2006, 356:182-193.)
The common ground of the methods focusing on error propagation is that they are based on a Gaussian approach. This also pertains to the methods using first-order Taylor expansion, due to being equivalent with the former. A required prerequisite for using Gaussian error propagation or first-order Taylor expansion is that the errors need to be very small. If this is not given, the error will propagate to a highly non-normal distribution, which results in the propagated errors not being a realistic estimate. This assumption is even more severely impeded by the fact that ratios in real-time PCR are based on exponential calculation, i.e. efficiencies to the power of threshold cycles. We analyzed the effect of the error distribution of the starting variables (efficiencies, threshold cycles) and define boundaries in which Gaussian error propagation is still feasible. The parametric approaches are compared to the permutation approaches as utilized by the REST software. For the case of 'large errors' we investigated two alternatives based on either Monte Carlo simulation or high-order Taylor expansion, both of which are computationally very demanding. For solving this task and supplying this very mathematic approach to the biological community, we introduce an *R* package with the corresponding functionality.

rtprimerdb.org: public qPCR assay database with custom assay quality control and primer design pipeline

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Real-time quantitative PCR is matured during the last decade and it became the leading technology for the detection and quantification of nucleic acids. We are at the beginning of a new era with the introduction of new techniques in sample preparation and handling and the evolution of the detection instrumentation which allows up scaling the number of samples and targets analysed during one experimental procedure. One of the last obstacles in such a high-throughput workflow is the lack of automation, standardisation and transparency in the design, validation and selection of assays. During this presentation I will outline the features of RTPrimerDB (http://www.rtprimerdb.org), a

freely accessible database and analysis tool for real-time quantitative PCR assays recently extended with a primer design platform. RTPrimerDB includes records with user submitted assays that are linked to genome information from reference databases and quality controlled using an in silico assay evaluation system. The primer evaluation tools intended to assess the specificity and to detect features that could negatively affect the amplification efficiency are combined into a pipeline to test custom designed primer and probe sequences. An improved user feedback system guides users and submitters to give practical remarks and details about experimental evaluation analyses. The database is linked with reference databases to allow the submission of assays for all genes and organisms officially registered in Entrez Gene and RefSeq. Records in RTPrimerDB are assigned unique and stable identifiers. The content is provided via an interactive web-based search system and is available for download in the recently developed RDML format (http://www.rdml.org) and as bulk export file. We further diversified RTPrimerDB with a primer design pipeline coupled with a specificity analysis and template sequence analysis (SNPs and secondary structure) which lets you select new high-quality assays with a significant success rate. This tool is available to detect targets in the majority of sequenced organisms. Future improvements of the pipeline include the possibility to design primers for specific applications such as intron/exon boundary assays, exon spanning assays and 3C assays, and expanding the number of available organisms.
RTPrimerDB is a one-stop portal for high-quality and highly annotated real-time PCR assays.

Wednesday 11th March 2009

Session: **qPCR NOS Session (1)**
Normalization & Optimization & Standardization
Chair: **J. Vandesompele & S. Bustin**
Lecture hall **HS 15**

Session sponsored by:

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A new qPCR assay for the detection of Clostridium difficile

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Clostridium difficile (CD)–associated infection (CDI) is a cause of a substantial and increasing proportion of hospital deaths, being responsible for more deaths than all other intestinal infections combined, rising by 72% between 2005 and 2006 to around 6,500 people in the UK alone. Symptoms range from mild watery diarrhoea to life-threatening intestine perforation. The economic impact of CDAD, largely attributable to the extended hospital stays incurred, is considerable, recently estimated to be at least $3.2 billion in the US and €3 billion a year in the EU. Most current forms of diagnosis, designed to detect the organism and screen for the presence of its toxins, are inadequate as they are slow, costly and poorly standardised. The high frequency of CDI, the likelihood of a poor clinical outcome for cases that are not treated promptly and the emergence of new more virulent CD strains, highlights the urgent need for more rapid and flexible, as well as less costly methods for detecting CDI and identifying the best course of treatment. A number of commercial PCR-based assays have been developed that detect the presence of bacterial toxin B. Their main drawbacks for clinical use are that they are very expensive, can require specialised instrumentation and provide limited information. We have developed a very cheap, but sensitive and specific assay for the simultaneous identification of several C. difficile targets as well as a host polymorphism reported to be associated with disease severity.

RealTime ready – Functionally Tested qPCR Assays for Gene Expression Analysis on the LightCycler® Platform

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Since their introduction in 1998, the LightCycler® Real-Time PCR Systems from Roche Applied Science have stood for maximum flexibility, high speed, and outstanding data accuracy. The LightCycler® 480 System continues this tradition and extends it to higher throughputs by using a plate-based analysis format. The unique LightCycler® 480 Software algorithms allow fast and highly accurate data generation, without sacrificing comprehensive versatility: e.g., basic and advanced methods for relative quantification. The increasing complexity of the 96- and even 384-well formats of the LightCycler® 480 platform, demands for simple and convenient content supply for large scale gene expression studies, employing the advantage of high dynamic range and sensitivity that only real-time PCR can provide. With the new RealTime *ready* assays, Roche Applied Science now offers a convenient and reliable solution of content supply specifically for the LightCycler® 480 System. RealTime *ready* assays are based on the unique Universal ProbeLibrary technology which allows fast and flexible assay design for millions of targets from virtually any organism. Each assay is extensively tested for sensitivity, PCR efficiency and signal intensity. Expert selected panels for pathways and relevant gene families are available with pre-plated, ready-to-use assays. Full customization of RealTime ready is planned for 2009. This will enable researcher to create their own panels on an easy-to-use online configuration portal or to order single assays for their targets of choice. Here we will provide performance data and scientific experimental data.

RDML: structured language and reporting guidelines for real-time PCR data

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Real-time PCR (qPCR) is the gold standard for quantitative gene expression analysis. Exchange of qPCR data between real-time PCR users or submission of data as Supplemental Material to a publication is cumbersome as all instruments have proprietary data collection software with a specific data format. To address this problem the Real-time PCR Data Markup Language (RDML) was developed (Lefever et al., Nucleic Acids Research, 2009). RDML is a structured, flexible and universal data standard, created to facilitate the data sharing across different information systems. The standard is intended to accommodate the storage of data from multiple experiments along with all related information regarding samples, targets and assays, and enables transparent exchange of annotated qPCR data between instrument software and third-party data analysis packages, between colleagues and collaborators, and between authors, peer reviewers, journals and readers. Importantly, to allow re-analysis of the data stored in an RDML file and to guarantee compliance with quality standards it is crucial that data acquisition, analysis and reporting become more standardized. Therefore, RDML files can hold all data and information required by the MIQE checklist (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) . MIQE is a collection of guidelines describing the minimum information necessary for reevaluation of real-time quantitative PCR experiments (Bustin et al., revised version submitted). To support the public acceptance of this standard, an on-line RDML file generator is made available to end users, and RDML software libraries supporting import and export of RDML data files have been created for software developers. All relevant information regarding the data standard, available tools and compliant software can be found on the RDML consortium website (http://www.rdml.org).

ZNA: new high-affinity synthetic oligonucleotides as powerful tools for PCR

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Zip Nucleic Acids (ZNAs) are new synthetic modified oligonucleotides, which improve hybridisation properties of nucleic acids. The rational approach of ZNAs was to increase the affinity of an oligonucleotide for its target by decreasing the electrostatic repulsions due to the polyanionic nature of nucleic acids. This was achieved by conjugating a polycationic moiety to the oligonucleotide. The automated synthesis of ZNAs uses classical phosphoramidite chemistry and allows customised introduction of cationic residues during oligonucleotide synthesis. Moreover, every standard oligonucleotide modification such as dye, quencher or tag can be easily incorporated into ZNAs. The melting temperature of ZNAs increases significantly and linearly with the number of cationic units grafted on the oligonucleotide, providing a convenient means for finely tuning hybridisation temperatures. Used as primers in real-time PCR assays, ZNAs demonstrate an exceptional high affinity for their target, work at low concentrations, at flexible annealing temperatures and circumvent salt concentration adjustments. Short dual-labelled ZNA probes perform efficiently and may become a promising alternative to TaqMan MGB probes. Easy-to-design, ZNAs are versatile and potent new tools for PCR. By relaxing design and avoiding time-consuming optimisation steps, we anticipate that ZNAs will be particularly useful for SNP genotyping, multiplex PCR, high-throughput analyses and amplification of tricky target sequences such as AT rich regions.

Importance of experimental design and sample QC for robust and meaningful QPCR results

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With QPCR as the gold standard for sensitive sequence detection and the desire to detect even smaller differences in gene expression it becomes more important to focus on good experimental design. Assessing sources of variance in the experiment and addressing those in the experimental setup is crucial for robust and meaningful results. The number and type of replicate samples, the influence of the sampling and sample preparation process as well as the variability of the reverse transcription step can have a dramatic effects on the downstream QPCR results.

Normalization of real-time RT-PCR data using an external RNA control

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We report a novel procedure for normalization of real-time RT PCR data using an external RNA control gene. The procedure is the first to add an external RNA control gene to tissue on a per-unit-weight basis. The accuracy, suitability and usefulness of the procedure were demonstrated in experiments involving a severe physiological challenge, anoxia. The expression of the internal RNA control genes (reference genes) β-actin, cyclophilin A and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was investigated in brain and heart of normoxic and anoxic crucian carp (Carassius carassius), a fish that can sustain anoxia for months. The expression of these genes differed significantly between experimental groups, especially in heart. Consequently, these internal RNA control genes were unsuitable for normalization of real-time RT PCR data. For example, in anoxic hearts, β-actin and geNorm (the geometric average of β-actin, cyclophilin A and GAPDH) failed to detect a 2.5-fold increase in the expression of the stress-response gene HSC70. Collectively, our data suggest that normalization of real time RT PCR data can be improved by adding an external RNA control to the samples.

Highly Accurate Quantitative Gene Expression Analysis without Use of pre-defined Normalizer Genes using Pattern Recognition Analysis

Dan Shaffer, Volker Vogel and Don-Paul Kovarcik
Daniel J. Shaffer, VP and Founder, Bar Harbor Biotechnology, US, daniel.shaffer@barharborbio.com

Lonza and Bar Harbor BioTechnology, Inc. have teamed together to bring life science researchers the StellARray™ Gene Expression System. This innovative system consists of GeneSieve™ Query, StellARray™ qPCR Arrays, and Global Pattern Recognition™ Analysis which completely interrelate. It allows scientists to perform a bioinformatics search to select their gene(s) or pathway of interest, measure gene expression or gene copy number of their samples using Real-Time PCR arrays, and determine the statistical significance of their data using a convenient web-based analysis tool.
By allowing the experiment to determine the normalizer, instead of having to choose normalizer genes such as housekeeping genes a priori, researchers gain novel insight into what their cells are telling them. These insights are revealed by the Global Pattern Recognition™ (GPR) Analysis Tool, a novel method to analyze real-time (qPCR) data. It permits researchers to identify biologically and statistically significant changes, in some cases less than 2-fold, in gene expression that would normally be missed. The presentation will describe the components of the system and show a selection of application data.

Optimisation and standardisation of sample preparation with the Bead-beating technology in q-PCR analysis.

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In the context of sample preparation and cell lysis, Bertin Technologies (France) has developed a technology dedicated to the homogenization and grinding of soft to hard materials. The goal is to improve the first critical step in any molecular biology process and follow the latest requirements of analysis equipments which have radically improved in terms of throughput, reproducibility, detection limits and linearity.
Following specific mechanical engineering studies of bead beating technology, a high speed figure-8 multidirectional motion gives shaking energy to the beads that grind/homogenize samples in sealed tubes. This patented solution Precellys plays a large part in the analyse chain of rapid method to extract and detect or quantify DNA, RNA or proteins.
Bertin and its partners have been investigating mechanical lysis with the Precellys bead beater vs. manual, chemical or sonicator methods. Three applications illustrate the contribution of this equipment to the improvement of the molecular biology process: DNA extraction from plants, DNA/RNA extraction from micro-organisms, and DNA/RNA extraction from tissues.
Bead beating technology was successfully evaluated in these applications and satisfied users in term of efficiency without degradation of the material, reproducibility, time and labour saving that are mains items to consider.

Session: qPCR NOS Session (2)
Chair: A. Nitsche & A. Stahlberg
Lecture hall HS 15

Session sponsored by:

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Increasing QPCR throughput: simple steps to speed up results whilst minimising variance.

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With the ever-growing popularity in the use of QPCR as a sensitive technique for quantifying DNA and RNA, there is an increasing demand for higher throughput of reactions and faster protocol times. There are many steps within the workflow of a reaction that can be optimised to reduce the overall time they take. Of particular interest to our research is improving the throughput and speed of validating siRNA knockdown of mRNA using QPCR. However, developing a faster QPCR assay has many challenges. In particular, the improvement in speed should not come at a loss of assay performance. A standard QPCR reaction takes approximately 1 hour 45 minutes to complete, but time savings can be achieved, by significantly reducing the dwell times at both the denaturation and annealing/extension steps of the reaction. Sufficient reduction of these dwell times can result in the completion of a QPCR protocol in less than 1 hour. In order to validate the performance of fast QPCR experiments, we investigated the effects of amplicon length, GC content and secondary structure and it was determined that these characteristics do affect the success rate of a reaction, and therefore should be taken into account when designing experiments. Other time saving steps can be taken, for example, we show how the addition of a thermolabile deoxyribonuclease during the reverse transcriptase step can remove contaminating DNA from the reaction. It removes DNA contamination just as effectively as the gold standard method, whilst reducing the time and effort inherent in traditional DNase treatment. We have also investigated ways to improve the reproducibility of a reaction, which ultimately cuts down on the number of repeated experiments required due to lack of confidence in the data. For example, we looked at the plastic consumables for QPCR and monitored the effects of evaporation through poor sealing. Our studies show that many data points can be lost from analysis due to incorrect sealing procedures and that a good sealing method resulted in a high level of reproducibility in the data. Therefore, by employing a number of simple yet effective measures, users can significantly reduce many of the variables inherent in the QPCR process and at the same time increase the speed to achieve results.

Significant difference or artefact of the method? - The impact of temperature performance of real-time thermocyclers on generated qPCR results

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Real-time qPCR has become a wide spread applied technique for determining gene expression levels over the past ten years. Yet very little attention has been paid to the thermal performance of thermocyclers in this process and the relation between physical temperature deviation and biological impact on the real-time qPCR results. Without taking the temperature variability parameter into account it is questionable if the higher or lower level of expression of the gene of interest in comparison to one or more reference genes is real or an artefact of the method. In this study a large number of thermocyclers has been evaluted on a worldwide basis and show that large variations in block uniformity and accuracy between different brands of real-time thermocyclers, but also within the same brand and even within the same model do occur. As illustrated by the biological results of qPCRs performed on these thermocyclers, it can be seen that when the same qPCRs, using identical reagents, oligos, plastics, DNAs and temperature protocols are performed on different thermocyclers, they lead to different results. So to be able to generate reproducible and trustworthy results, also in intra- and interlaboratory studies, all parameters of the (RT)(q)PCR process, like the DNA/RNA , RT reagents, PCR reagents, oligos, but also the plastics and the thermocycler need to be optimized or controlled. Only then it is valid to matematically analyze, standardize and normalize data to show significant differences and not artefacts of the method.

The Next Generation in Hot Start PCR - CleanAmp Primers and dNTPs

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PCR is widely used for applications that require a high level of specificity, reliability, and reproducibility. While many solutions for improved PCR performance target the DNA polymerase, this talk will present approaches in which the dNTPs or the primers are modified. Studies will describe the concept of thermolabile dNTP and primer protecting groups as applied to Hot Start activation schemes in PCR. Further content will describe the application of these temperature-sensitive components into more advanced PCR applications such as Real-time PCR, multiplexed PCR, and fast thermal cycling PCR. The development of these orthogonal approaches for improved nucleic acid amplification provide great potential to be used alone or in conjunction with other PCR enhancement technologies.

Assessment of the reliability of nucleic acid extraction systems commonly used to get valid qPCR results

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The specimen preparation procedure preceding the reverse transcription/amplification is crucial important since errors occurring here can lead to false negative PCR results and inadequate treatment of the patients. Moreover, extensive loss of nucleic acid during the nucleic acid purification impairs the validity of the final qPCR results. To assess the recovery efficiency of various nucleic acid extraction systems commonly used today, we prepared aliquots of well-characterized samples containing different virus strains purchased from National Institute for Biological Standards and Control (NIBSC). Dilution series were prepared from these HIV-, HAV-, HBV-, or HCV WHO International Standards having calibrated titres of each of the viruses. Initially the negative human plasma used as diluent was spiked with a tailored liposome-based internal control vehicle, IC particles, to enable virus-independent comparison and whole process quality assurance of the analysis. Subsequently, the extraction of aliquots of the dilution series were carried out in the various nucleic acid purification technologies according to the manufacturer's instruction except that the volumes of the specimen input and nucleic acid extract were matched between the systems for comparison purposes. Both manual and automatic nucleic acid extraction systems were examined. Testing of the resulting RNA and DNA extracts was done in commercially available RT-qPCR and qPCR assays for each of the viruses. From the quantification standards included in the assays, the recovery of each of the nucleic acid extraction system was calculated. The presentation will disclose which extraction platforms are the best for these clinically important viruses.

Housekeeping genes validation in acute and chronic adjuvant arthritic rat for mRNA quantification by real time RT-PCR

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Real time RT-PCR is one of the efficient methods in mRNA quantification. One of the approaches for quantification is the normalization of mRNA by internal control gene or housekeeping gene. The expression level of internal control should remain constant in different tissue types and in different experimental conditions for efficient quantification. In the present study we have compared the expression levels of 10 commonly used housekeeping genes in 9 different tissue types in normal, acute and chronic adjuvant arthritic conditions in rat. We have also studied CD3 gene expression in ankle joint and cortical bone to evaluate the differences in the levels of housekeeping gene expressions due to inflammatory cells. Our data showed an increased of all HKGs in acute and chronic adjuvant arthritis. HPRT was the most stable housekeeping gene among all the tested ones in acute and chronic adjuvant arthritic conditions in most of the tissue types in rat except for the ankle joint and cortical bone. There was no stable HKG in both the tissues in acute and chronic condition. We have also found a significant increase in CD3 gene expression which was significantly correlated with the increased expression level in ankle joint and cortical bone in both acute and chronic adjuvant arthritic rats. In conclusion our data suggested HPRT to be the most stable housekeeping gene in most of the tissue types in adjuvant arthritic condition in rat. Further more an increase in the level of housekeeping gene expression in ankle joint and cortical bone is due to the increase of inflammatory signatures in acute as well as in chronic adjuvant arthritic rats.

Gene Expression Analysis by Genome Controlled Reverse Transcription-PCR.

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Genome controlled reverse transcription PCR (gc-rtPCR) is a technique for the quantitative analysis of multiple transcripts in mammalian cells. In gc-rtPCR, primers used for the reverse transcription step are designed to alter the sequence of the cDNA compared to the genomic sequence, and change the Tm of the resulting PCR product by 3-4 degrees. During the subsequent PCR step, both the genomic DNA and the cDNA are co-amplified using a single pair of primers, and in-tube melting curve analysis is then used to quantitate the relative abundance of the cDNA vs. the genomic DNA. The technique requires only a small amount of tissue and uses small 50-65 bp amplicons, and thus is suitable for a variety of starting materials, including highly fragmented mRNA in FFPE samples. The technique has a detection limit of 100 copies of cDNA, and a dynamic range of 4 orders of magnitude. Accuracy and reproducibility analyses compare favorably to TaqMan analysis of the same genes. The uniplex nature of the amplification reactions in the gc-rtPCR technique enables flexible combination of multiple wildtype and control genes in the same assay. This novel technique provides a robust platform for multigene predictor applications.

QPCR Use in Biopharmaceuticals and Current Issues
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Brief overview for uses of QPCR in Biopharmaceuticals, (covering mAbs, DNA vaccines, & SCVs etc) starting with candidate selection, aid to cell line selection, aid to cell line stability issues, use as a potency assay for both DNA vaccines and mAbs (by use of Biomarkers), Process viral clearance validation, Process genomic DNA clearance, Cell bank testing, Biodistribution, biomarker analysis in clinical trials, and final it's potential in companion diagnostics. The end of talk will highlight current, general, issues with the QPCR technology, current regulatory guidance issues and the requirement to standardize on things like analysis methodology and algorithms (it's a regulatory must) and future considerations for platform vendors in both software and hardware.

Closing of the Symposium
Lecture hall HS 14

17:30 **Closing of the Symposium**
Heinrich HD. Meyer & Michael W. Pfaffl

Abstracts - Poster presentations

P001

qPCR BioStatistics & BioInformatics
Location: poster room (mensa)

A Rapid Bioinformatic Engine for Multiplexed qPCR Design
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Multiplexed qPCR remains a challenging endeavor for reasons that include: 1) designing assays to combine without interference, 2) resolving fluorophores using the optics of each real-time instrument, and 3) optimizing and validating each assay's performance. Here, we address each of these issues when developing several pentaplexed assays that target genes from human and mouse. Each assay was designed using a free, online, software program that carefully considers inter-oligo interactions while simultaneously building its multiplexed set. Situations of disproportionate copy number present a particular challenge upon multiplexed performance; additional validation is needed to define the limits of a multiplexed set, as compared to individually amplified assays.

P002

BCI: an R-based algorithm for computing bootstrap-t confidence interval for nucleic acids concentration by absolute real-time PCR
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There are two major approaches of real-time PCR quantification: the absolute and the relative method. The latter evaluates the change in expression of the target gene relative to a reference gene, whereas the absolute method uses a standard curve to quantify unknown amount of nucleic acids in a target sample. From a statistical view point, the standard curve corresponds to the simple linear regression model and thus the absolute quantification method allows to exploit the methodological background of the linear regression theory. By means of a technique known as inverse regression, the fitted standard curve is used as calibrator to estimate the unknown nucleic acid concentration in the target sample. Several approaches have been proposed for constructing confidence intervals in inverse regression. We propose an user-friendly algorithm, named BCI (Bootstrap Confidence Interval), specifically designed to compute bootstrap-t confidence interval for nucleic acid concentration by absolute real-time PCR. The algorithm has been written in R language an open-source statistical software and provides the bootstrap estimate of the unknown concentration both in logarithmic scale and in its original scale as copy number together with the lower and upper limits of the 100(1-α)% bootstrap-t confidence interval of the unknown concentration. Users can modify the number of bootstrap resampling and the confidence level (1- α) of the bootstrap-t confidence interval.

P003

Data analysis for gene quantification and expression profiling using GenEx.
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As the data size and complexity from qPCR projects increase, the need for comprehensive automated or semi-automated software tools increase rapidly. Software tools can provide support after data collection by providing data pre-processing, statistical analysis and visualization capabilities, often for hypothesis generating purposes. Alternatively they can be used prior to experimental realization by helping to define experimental design parameters for hypothesis validation assays. The GenEx software from MultiD Analyses AB provides all of the capabilities mentioned above. Performing accurate qPCR data pre-processing is very important, particularly for quantification purposes. Many steps are usually implemented and it is useful to follow protocols in order to avoid introduction of unwarranted processing variability and bias. The protocol available in GenEx is easy to adapt to user-specific needs while at the same time comprehensive enough to enable users to easily perform accurate pre-processing. As scientists we believe observations we make are manifestations of rational processes. Our challenge is to identify the particular rational process that we want to study while minimizing contributions from rational processes that would obscure the understanding of our particular study. Contributions from unwanted processes are often called random although the underlying processes may not be. Tools to differentiate between contributions from desired and unwanted processes include parametric and non-parametric statistical tests, scatterplots, principle component analyses and neural network analyses. These tests and analyses and more are available in GenEx. Validation of scientific conclusions is not absolute, but based on reproducibility. No scientific theory is above scrutiny and potential revision. However, based on certain assumptions, an increasing number of observations that supports a particular conclusion will also increase our confidence that the

particular conclusion is going to continue to be supported by future observations. We may thus define a level of confidence by which we would assign our conclusion to be "true". A good experimental design aimed to validate a hypothesis should therefore include the number of necessary observations needed to obtain the desired level of confidence, before realization of the experiment. Based on a hypothesis of an observed effect, including desired level of confidence, amplitude of desired observed effect and variability of confounding effects, GenEx can calculate the number of necessary observations. The presentation will focus on important considerations for running qPCR experiments and ways the GenEx software may provide support.

P004

An alternative way for Real Time PCR data Analysis

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INTRODUCTION - Real Time PCR is a methodology with increasing applications in the clinical laboratory. This new and revolutionary method combines the PCR chemistry with the fluorescent probe/dye detection of the amplified product, all in the same reaction tube. Since the equipment used can record the emission of fluorescence during all the cycles of amplification, a significant increase of the PCR product is directly linked with the initial amount of target DNA. In Real Time PCR, we can determine a fixed fluorescent treshold, above the background. When the PCR product that we want to detect cross this threshold, we can determine a parameter named Cycle Threshold (Ct). All the equipment used in Real Time PCR experiments have some kind of software to analyse the data, namely the analysis of the expression of Ct value relatively to the log[DNA]. However, this software doesn't give much details regarding the linear regression: it only calculates the slope, y intercept and coefficient of determination (R2). AIM - Development of an Excel sheet that calculates several parameters regarding the linear regression and assay validation/calibration. RESULTS - We have developed an Excel sheet that uses the data from calibration data (4 different concentrations of Virus) in order to determine the following parameters:
• Slope
• Y Intercept
• Coefficient of Determination
• Efficiency Amplification
• Detection and quantification limit (analytical and method)
• Standard Error (RMSE)
• P-Value associated with the linear regression (validation)
Relatively to the linear regression parameters, we have introduced the ANOVA (Analysis of Variance) in order to determine the Sum of Squares of: Regression, Residual, Lack of Fit and Pure Error. With this approach we can make an objective analyses of the goodness of fit, residuals, determine outliers and the confidence interval of the samples with viral load. Since our laboratory is accredited (ISO 17025: 2005), it also helps in the maintenance of records relatively to batch/expire date of DNA/RNA extraction kit, primers, probes, master mix, internal control (amplification) and the reaction plates. CONCLUSION - This Excel sheet is a very good alternative to the data analysis of the standard software present in the various Real Time PCR equipments.

P005

RefGenes: new tool to find suitable reference genes

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Reference genes (or "housekeeping genes") are often used as internal controls for transcript quantification assays. Often, classical reference genes such as GAPDH or TUBB are not suitable for one's own experimental condition because their expression varies significantly. The goal of RefGenes is to identify, from a genome-wide set of genes, those that are most suitable for a given condition. This is achieved by screening Genevestigator's large expression compendium (>27,000 microarrays). Validation experiments on plant and animal qRT-PCR experiments showed that genes found through RefGenes performed significantly better as normalizers than classical reference genes.

High throughput quantitative PCR
Location: poster room (mensa)

P006

A High Throughput, Quantitative Real Time PCR Method for the Determination of Copy Number Variation in Knockout Mice

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Knockout mice are important tools in studying gene function and investigating genetic disorders as complete loss of gene function is established. A knockout mouse is generated by replacing both alleles of a target gene within an embryonic stem cell. This is done using a gene trap vector to generate a chimera (F1), selective breeding results in F2 progeny and subsequent knockout mice (F3). At each stage a combination of genotypes is possible. The European Conditional Mouse Mutagenesis programme (EUCOMM) and the Knockout Mouse Project (KOMP), are utilising this approach to knockout ~ 20,000 genes, to provide a public resource of thousands of knockout mice by 2011.
A major challenge when generating knockout mice is genotyping the F1-F3 progeny. The current method of screening these mice involves sequencing and long range PCR. The former technique is time consuming and laborious where as the latter technique is unable to accurately distinguish between the different genotypes. We have developed a rapid and accurate high throughput quantitative real time PCR method to determine accurately genotype transgenic mice and their progeny. Furthermore, this high throughput approach can also be used to study human copy number variation. Genetic variation and in the human genome can cause susceptibility or resistance to disease. Human copy number variation has been associated with a variety of diseases such as cancer, HIV infection, inflammatory autoimmune disorders, autism and schizophrenia.

P007

TaqMan® OpenArrayTM: A Breakthrough System For Nano-Well High Throughput Genotyping

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Molecular research and analysis in the field of plant and animal genomics is rapidly developing and expanding into new application areas, requiring the development and introduction of new technologies, assays and tools to support his research. Within this broad field of research, marker-assisted selection, QTL mapping and backcross analysis are widely used techniques to identify genes, loci and polymorphisms encoded into the genomes of all living organisms. Here we present the TaqMan® OpenArrayTM system, a breakthrough technology uniquely positioned for these kinds of applications. By combining gold standard TaqMan® SNP Genotyping assays with the efficient nano-reaction OpenArray™ platform, researchers are able to genotype large numbers of samples over any customizable set of markers with a single platform instrument. The TaqMan® OpenArray™ system utilizes 3072 nano-well plates, innovative fluidic properties and a one day protocol to generate high quality, robust genotype profiles. This platform is especially enabling for researchers who have continuous streams of incoming samples that require a quick and cost-efficient workflow. To demonstrate the capabilities of this platform, we have genotyped several hundred single nucleotide polymorphisms across a panel of genomic DNA samples and report the performance in terms of assay pass rate, sample call rate and concordance to conventional TaqMan® SNP Genotyping. In addition, innovative solutions for research projects with sample quantity limitations have recently been developed and will be presented here for review. In summary, we will discuss how this system differentiates itself from the current genotyping technologies.

P008

QuantPrime - a flexible tool for reliable high-throughput primer design for quantitative PCR

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Medium- to large-scale expression profiling using quantitative polymerase chain reaction (qPCR) assays are becoming increasingly important in genomics research. A major bottleneck in experiment preparation is the design of specific primer pairs, where researchers have to make several informed choices, often outside their area of expertise. Using currently available primer design tools, several interactive decisions have to be made, resulting in lengthy design processes with varying qualities of the assays. Here we present QuantPrime, an intuitive and user-friendly, fully automated tool for primer pair design in small- to large-scale qPCR analyses. QuantPrime can be used online through the internet (http://www.quantprime.de/) or on a local computer after download; it offers design and specificity checking with highly customizable parameters and is ready to use with many publicly available transcriptomes of important higher eukaryotic model organisms and plant crops (currently 295 species in total), while benefiting from exon-intron border and alternative splice variant information in available genome annotations. Experimental results with the model plant Arabidopsis thaliana, the crop Hordeum vulgare and the model green alga Chlamydomonas reinhardtii show success rates of designed primer pairs exceeding 96 %. QuantPrime constitutes a flexible, fully automated web application for reliable primer design for use in larger qPCR experiments, as proven by experimental data. The flexible framework is also open for simple use in other quantification applications, such as hydrolyzation probe design for qPCR and oligonucleotide probe design for quantitative in situ hybridization. Future suggestions made by users can be easily implemented, thus allowing QuantPrime to be developed into a broad-range platform for the design of expression assays.

P009

Evaluation of Digital PCR for Absolute Quantification

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Digital PCR involves diluting and partitioning a sample between many hundreds or thousands of individual PCR reactions such that a single molecule or less on average is present in each reaction. Determination of the number of positive amplifications is indicative of the number of targets present in the sample. As such, digital PCR does not suffer from inaccuracies that often arise from standard real-time PCR approaches at trace levels using calibration curves, for example through extrapolation, and it affords the potential for absolute quantification. Recent advances in microfluidics have facilitated the development of digital PCR by combining microfluidics with single molecule, nanolitre volume PCR to increase levels of replication, throughput and cost efficiency.
In this study we have evaluated the performance of digital PCR for absolute quantification using the Fluidigm BioMark Integrated Microfluidics System as compared to standard real-time PCR using the ABI 7900HT system. The performance of the systems was evaluated using a DNA standard of known molecular weight. The ability of the two platforms to discriminate changes in copy number was assessed using different ratios of RNA standards in a background of human total RNA. Sensitivity was investigated using different starting amounts of the RNA standards.
The results of this study are presented here and demonstrate the potential of digital PCR approaches for absolute quantification. They also highlight some of the areas of uncertainty and variability that are inherent with this approach.
Acknowledgements:
This work was supported by a grant from the Department of Innovation, Universities and Skills (U.K.) under the National Measurement System Chemical and Biological Metrology Programme.

P010

PERFORMANCE OF THE THERASCREEN K-RAS MUTATION ASSAY ON THE LIGHTCYCLER480®Type II INSTRUMENT IN A CLINICAL ROUTINE SETTING

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Objectives : Recent approval of Vectibix® (Panitumumab) fully humanized monoclonal antibody therapy for advanced colon cancer created an urgent need in hospital laboratories to screen K-Ras oncogene mutations. We assessed the LightCycler® 480 instrument together with the Therascreen K-Ras mutation-specific scorpion primer PCR assay for its practicality, easiness of use and performance in a medium sized routine hospital laboratory setting.
Methods : 40 genomic DNA samples were extracted from three 10 micrometer thick paraffin sections of colorectal tumor tissue using a QIAamp®DNA Mini Kit (QIAGEN). Prior proteinase K digestion was performed according the company instructions (DxS document GEN/023/2). Each section was pre-selected by a pathologist and checked for > 70% tumor tissue content. Final dissolved DNA content was measured with a Quant-IT dsDNA BR Assay Kit on a Qubit fluorometer (Invitrogen) or a Biophotometer (Eppendorf). 20 ng purified DNA were used for the K-Ras Strip Assay™ (ViennaLab). 20 ng purified DNA samples were used for the Therascreen K-Ras assay (DxS). The necessary assay adjustments for the LightCycler480®Type II instrument are described in the technical notes (DxS codes KR-03/04) and in the colour compensation reagents instructions for use. Crossing points (CPs) were determined with the second maximum derivative function of the absolute quantification module (LightCycler® 480 software 1.5).
Results : We characterized PCR performance parameters of the internal K-Ras exon 4 amplification control reagent and two external control genes (beta-actin and 18SRNA) on the LightCycler480® system. PCR efficiences on the formaldehyde-fixed and paraffin-extracted genomic DNAs (n=10) were high: beta-actin (1.93), 18SRNA (2.07), K-Ras (1.95). Intra-assay variabilities were small: beta-actin (CP: 31.73±0.81; CV= <1.50), 18SRNA (CP: 19.75±0.34; CV= < 0.31), K-Ras (CP: 28.47±0.52; CV= <0.5). The inter-assay variability (CV% of three assays performed within a month) of the mutation specific standard mixtures CPs within a Therascreen kit lot (version 2) was as follows: Gly12Ala: 0.52, Gly12Asp: 0.92, Gly12Arg: 1.35, Gly12Cys: 1.38, Gly12Ser: 2.2, Gly12Val: 0.24, Gly13Asp: 1.78. The results of the DxS real time PCR assay corresponded 100% with externally tested samples (n= 20) and with the K-Ras Strip Assay™ (n= 10). In two samples a double mutation (12Val/12Asp) or multiple mutations (12Ala, 12Cys, 12Ser, 13Asp) were determined.
Conclusions : The Therascreen K-RAS assay performed on the LightCycler480® instrument is a reliable technique for accurate mutation detection on paraffin extracted genomic tumor DNA. Its major advantages when performed within a clinical setting are the work time savings (no need for post-PCR processing) and a minimized risk for a laboratory PCR product contamination due to foil-sealed PCR plates.

P011

Analysis of gene expression profile by cDNA microarray and qPCR in fibroblast cultures of ALMS patients.

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Introduction: **Als**tröm Syndrome (ALMS) is a rare, autosomal monogenetic disease, caused by mutation in *ALMS1* (Chr 2p13), a gene ubiquitously expressed with unknown function. ALMS shows impairments at multiple organ systems, resulting in blindness, hearing impairment, childhood-onset obesity, hyperinsulinemia, insulin resistance and type 2 diabetes. A very common feature of ALMS patients is an extensive fibrosis evident at multiple anatomical site: kidney, heart, lung, liver, pancreas, bladder, ovary and testis. This fibrotic substitution can cause specific and severe organ failure bringing often to an early death.

Aim: we established fibroblast primary cultures from dermal biopsy of 4 ALMS patients and 3 control subjects. The cells were employed to analyze the modulation in gene expression profile related to the presence of mutated *ALMS1* transcripts.
Research design and methods: we investigated in primary fibroblast cultures the gene expression profile by cDNA microarray analysis. RNA deriving from 4 ALMS patients was co-hybridized with the RNA pool obtained from 3 healthy controls, using an oligo-spotted microarray platform. We quantified the collagen-specific genes expression by qPCR .
Results and conclusions: From about 21500 genes represented, 188 resulted as up-regulated whereas 372 were identified as down-regulated in ALMS patients. Gene function was evaluated and modulated transcripts were clustered in main categories such as “extracellular matrix component”, “cell cycle”, and “apoptosis”. Data analysis showed an up regulation of numerous collagen transcripts. The real-time PCR quantification confirmed ALMS fibroblasts express higher level of mRNAs coding for different type of collagens (in particular COL8A1 and COL15A1), suggesting an involvement of *ALMS1* in the multi-organ fibrosis.

P012

A novel digital technology for non-enzymatic direct multiplexed measurement of gene expression

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We describe a novel technology, the nCounter system, for highly multiplexed analysis of gene expression levels. Then nCounter system detects individual mRNA molecules using an assigned code sequence of fluorescent molecules, and counts the number of times that code appears in a sample. No enzymes are used in our system; rather, the collection of probes is hybridized in solution to RNA in a sample. Experiments performed in a single multiplex analysis of 550 human genes revealed a correlation coefficient of 0.999 between replicate measurements, a detection limit between 0.1fM (0.2 copies/cell) and 0.5fM (1 copy/cell), and a linear 500-fold dynamic range. The nCounter system can detect a 1.5-fold increase or decrease in expression across a broad range of expression, and as little as 20% changes in expression for genes present between 1fM and 10fM. We demonstrate a good correlation between nCounter system and Affymetrix GeneChip technology, and better correlation with TaqMan, for –fold change measurements using two different experimental paradigms. Furthermore, a comparison of transcript levels measured by the nCounter system with SYBR green RT-PCR demonstrated a high correlation in the gene expression pattern at all transcript levels. We show that a whole cell lysate can be used as starting material with equivalent results to purified total RNA. Finally, we show that RNA extracted from formalin-fixed paraffin embedded (FFPE) tissues can be used in the nCounter system to analyze expression levels in archived samples. Our unique direct detection and digital quantification approach results in unprecedented sensitivity, precision and reproducibility in gene expression analyses. Materials & Methods - nCounter hybridization reactions were performed in triplicate with total RNA samples isolated from mock and polio virus infected human A549 cells. nCounter reactions were set up as follows: 100ng of total RNA Reporter and capture probes for 509 human mRNAs and controls made to non-human sequences (6 positive, 2 negative) DNA control targets spiked in at 0.1, 0.5, 1, 5, 10 and 50 fM Hybridizations were carried out for 20h at 65°C. Excess reporters were then removed by using magnetic bead based purification. The same samples and amount of RNA were also analyzed with Affymetrix® U133Plus2 arrays, using the two-cycle amplification/labeling protocol recommended by the manufacturer. We selected a subset of 14 genes in which the measured log2 fold-change was significant in one platform but not the other for further analysis by TaqMan Real-Time PCR. In a second experiment, nCounter hybridization reactions were performed in triplicate as described above with total RNA samples isolated from sea urchin embryos collected at seven different development time points. A set of 21 genes were selected for comparison with existing SYBR Green Real-Time PCR data generated in the Davidson Lab.

P013

DNA amplification in flow-through microreactor

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The work presents a microfluidic chip system with optimized thermal and fluidic characteristics for flow-through polymerase chain reaction. The designed microreactor comprises a heating plate consisting of 5 temperatures zones and a fluidic chip with meandering microchannels. The optimized thermal profile allows the implementation of one PCR cycle in a half channel loop. With this condition the microreactor possesses a 40 cycles flow-through thermocycler on the footprint of a microscope slide. In addition the microfluidic chip system was designed to operate at segmented-flow conditions for high-throughput analysis of PCR samples in a small volume of 10 – 100 nl. Each droplet of PCR solution in a flow of mineral oil may contain a single sample that is independently processed while transported through the microchannel. The surface of the microchannels was chemically modified to assure stable fluidic conditions. The PCR conditions can be adapted to extensive applications by variation of the flow-rate and the geometry of the temperature zones. The system is designed for an application in point of care tests and as a part of a system for the control of a fermentation process. Due to the sensitivity of the PCR process to contaminations the use of disposable microchannel devices is commonly preferred. Disposable chip devices made from polycarbonate were tested within the flow-through microreactor for the detection of the tumor suppressor gene p53 and compared to the functionality of all-glass chip devices. Although product yield and selectivity of the PCR process do not depend on material of the microchannel devices, a well defined and reliable segmented flow could only be realized in the all-glass microchannel device. The MINAMED-project is supported by grants of the BMBF (16SV3529).

P014

PCR and microarray chip technologies for Phytophthora diagnosis

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The polymerase chain reaction (PCR) has been established as a standard method in molecular biological analysis for amplification of small amounts of nucleic acids. Due to the amplification of the target DNA their detection becomes easier or even only possible. Therefore, electrophoresis, real-time PCR or hybridization based assays using DNA microarray technology are applied.
Recently, we developed a stationary PCR chip device for fast DNA amplification using minimal volumes of reaction mixture with low power consumption. For example the amplification of a 131 bp fragment can be performed within 23 min including 45 PCR cycles. Heating and cooling ratios of up to 15 K/s are realized and the power demand amounts to approximately 3 watts. The rapid operating speed results from low volumes of reaction mixture (down to 0,5 µl) that are applied on the chip surface where thin film platinum layers are located that act as heating structures and temperature sensors. Special disposable glass slides are utilized as sample carriers to realize single use only in order to avoid cross contamination. A miniaturized device including the stationary PCR chip and all elements for control is already developed. This portable device includes a PDA (personal digital assistant) for parameter input and control as well as rechargeable batteries that provide sufficient power for one day of operation. A fibre-optics based extension is available for real-time monitoring of the amplification process that enables quantization of the target DNA. Thereby time consuming DNA analysis following the amplification

steps are eliminated. For this purpose different fluorescence based detection methods with SYBR Green as intercalator or a specific TaqMan probe were applied. Using the stationary PCR chip a total amount of only 2 DNA-molecules/µl could be reproducibly detected. Current and future works are focused on integration of the PCR process and DNA-chip-technology for readout of information using a single device. Caused by the disadvantages of fluorescence readout such as complicate detection equipment that is also bulky and expensive we use a reliable electrical detection system based on enzymatically catalysed, reductive silver enhancement that bridges microelectrodes. For this purpose a DNA chip with integrated electrode gaps was developed. Our aim is the development of an analytical system for point-of-care diagnosis of the phytopathogen Phytophthora that causes decay of several plants such as soya, oak, potato or tomato.
This work is supported by grants of the Federal Institute of agriculture and food (support code: 28-1-42.027-06).

qPCR NOS Session
Location: poster room (mensa)

P015

Oligo Design Across the Mouse Genome
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Fluorescence-quenched probes are routinely used to gauge gene copy number. We describe a bioinformatic engine for the design of such oligos, and used to generate five thousand TaqMan assays for the NIH Knockout Mouse Project (KOMP). Here, we demonstrate the performance of a subset when amplified upon wild-type mouse gDNA. Analysis of this data-set uncovers important trends in amplification performance and emphasizes the need to screen assay specificity using both bioinformatic and empirical approaches. Redundancy and accessibility are considerations that become pronounced in large-volume sequence design. Based on this experience as well as user feedback, new software functionality is introduced to improve upon these qualities.

P016

Selection of reference genes for qRT-PCR examination of wild populations of Atlantic cod Gadus morhua
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Background: Extensive sequencing efforts have been taking place for the Atlantic cod (Gadus morhua) in recent years, the number of ESTs in the Genbank has reached more than 140.000. Despite its importance in North Atlantic fisheries and potential use in aquaculture, relatively few gene expression examinations exist for this species, and systematic evaluations of reference gene stability in quantitative real-time RT-PCR (qRT-PCR) studies are lacking.
Results: The stability of 10 potential reference genes was examined in six tissues of Atlantic cod obtained from four populations, to determine the most suitable genes to be used in qRT-PCR analyses. Relative transcription levels of genes encoding β-actin (ACTB), elongation factor 1A (EF1A), actin-related protein-2 (ARP-2), glyceraldehyde-3P-dehydrogenase (GAPDH), ubiquitin (Ubi), acidic ribosomal protein (ARP), ribosomal protein S9 (S9), ribosomal protein L4 (RPL4), RPL22 and RPL37 were quantified in gills, brain, liver, head kidney, muscle and middle intestine in six juvenile fish from three wild populations and from farmed Atlantic cod. Reference gene stability was investigated using the geNorm and NormFinder tools. Based on calculations performed with the geNorm, which determines the most stable genes from a set of tested genes in a given cDNA sample, ARP, Ubi, S9 and RPL37 were among the most stable genes in all tissues. When the same calculations were done with NormFinder, the same genes plus RPL4 and EF1A were ranked as the preferable genes.
Conclusions: Overall, this work suggests that the Ubi and ARP can be useful as reference genes in qRT-PCR examination of gene expression studying wild populations of Atlantic cod.

P017

Validation of housekeeping genes for gene expression studies in human ejaculate
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BACKGROUND: Beta-actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), heat shock protein (HSP) and ATP-synthase subunit 5B (ATP5b), with distinct functional characteristics and expression patterns were analyzed in use as references for gene expression profiling using quantitative Real-Time PCR (qRT-PCR) in human ejaculate. OBJECTIVES: To determine the expression stability of 4 commonly used reference genes in the ejaculate of fertile and infertile patients. METHODOLOGY: Semen was evaluated using standard World Health Organization (WHO) procedures. Each assay included a standard curve (exponent base 10) of four serial dilution points of cDNA (raging from 5 ng to 0.005 ng). All samples were run in duplicate and the mean value of each duplicate was used for all further calculations. The stability of selected reference genes was analyzed using the GeNorm software. RESULTS: Calibration curves were generated using relative concentrations vs. threshold cycles (Ct). The RSq value (R2, linear correlation coefficient), an indicator of fit for the standard curve plotted to the standard data points of all genes ranged from 0.982 to 1.000. Based on the slopes of the standard curves, the mean of the amplification efficiencies were above 97%. The genes showed different expression between fertile and infertile patients. The Ct deviation averages were GAPDH (2.17), beta-actin (2.32), HSP (1.053), and ATP5b (3.34). GeNorm identified GAPDH and beta-actin as the most stable pairwise variation of reference genes for the infertile ejaculates (M value for combination of best two genes were 0.148). CONCLUSION: In infertile patient samples, a combination of GAPDH and beta-actin yields stable reference gene expression levels, whereas the use of HSP or ATP5b is not suitable for normalization of qRT-PCR results in this sample type. Our results provide information an appropriate reference gene for the normalization of qRT-PCR data in the ejaculate necessary for future gene expression studies.

P018

Importance of RNA integrity assessment in a qRT-PCR workflow
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Real-time quantitative PCR (QPCR) is a highly sensitive method to assess gene expression changes in biological systems. As for all experimental designs, high quality starting material is essential for the success of the experiment. There are various mechanisms by which RNA can be degraded either at the 5' or 3' end. Not knowing the extent of possible degradation can lead to false negative results or misinterpretation of data if the amplicon falls into a degraded region. Therefore, the degradation level of RNA samples is an important parameter to monitor when designing primers and probes for QPCR. Here, it's shown how on-chip electrophoresis combined with a special RNA Integrity Number (RIN) algorithm can be used to assess the level of degradation of the RNA starting material. The results of the experiments indicate that the amount and directionality of degradation are highly gene-dependent and that the most pronounced effects appears below a RIN of 4.6.

P019

Comprehensive mRNA Profiling of Lipid-Related Genes in Microglia and Macrophages Using TaqMan Arrays and TaqMan Express Plates
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Cellular lipidomics is defined as the analysis of metabolism, transport, and localization of lipids species within cells. The quantitation of different lipid species from various biochemical pathways and biochemical analysis of lipid metabolism enzymes is an integral part of this concept. Recent progress in the field of transcriptomics, mainly the cost reduction of DNA-microarrays and the development of high-throughput real-time reverse-transcription (RT)-PCR systems have also enabled researchers to perform a comprehensive transcriptomic analysis of all lipid-related genes.
Here we describe the quantitative analysis of 41 selected lipid-related transcripts using a novel "Lipidomic" TaqMan Array. The TaqMan Array is based on an Applied Biosystems 7900HT microfluidic card. This method allows simultaneous analysis of 41 lipid-related genes and 7 controls in 2 replicates of 4 different samples per run. In addition, we analyzed the identical "Lipidomic" gene set and identical RNA samples on the recently launched "TaqMan Express Plates" – customizable 96-well plates with pre-spotted TaqMan Gene Expression Assays.
Our special interest was to study the expression of "lipidomic" genes in macrophages and microglia under conditions mimicking sterol loading and pro-inflammatory activation.
The TaqMan Array results show that (i) stimulation with the liver-X-receptor (LXR) and retinoid-X-receptor (RXR) ligands T0901317 and 9-cis retinoic acid (RA) induces several genes of lipid metabolism, (ii) lipopolysaccharide (LPS) and interferon-g (Ifn-g) strongly repress lipid-related genes, and (iii) co-incubation with docosahexaenoic acid (DHA) dampens the repressing effect of LPS. Our results were confirmed by the data obtained with the TaqMan Express Plates.
The method described here can be used to rapidly and accurate quantify transcriptionally dynamic "lipid" genes in any cell type. The "lipidomic" TaqMan Assay Set may be applied to study lipid disorders or to quantify the transcriptional effects of pharmacological treatments on lipid-related genes.

P020

RNA quality Indicator (RQI) - A New Tool for Assessing RNA Integrity to reliably detect differences in gene expression using RT-qPCR Experiments.

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RNA quality plays a major role in the generation of accurate quantitative results from gene expression analysis experiments. cDNA made from RNA that has been degraded will not become amplified to the same degree as cDNA made from intact, undegraded RNA. This can lead to erroneous conclusions regarding levels of gene expression when comparing samples that are degraded to different extents. To examine the effects of RNA degradation on quantitation of specific gene transcripts, qPCR was performed on equivalent amounts of RNA that had been degraded to various extents. The detection of amplified product was seen at successively later cycles as the RNA was degraded over time. The CT values of the qPCR reactions from five gene transcripts (18S rRNA, β-actin, β-tubulin, HPRT and GADPH) showed different degradation rates. Comparing qPCR results derived from RNA in different states of degradation will generate very different quantitative conclusions. This can be as great as 1000 fold, with samples subjected to 7 hr of heat degradation. The Experion automated electrophoresis system (Bio-Rad Laboratories, Inc.) provides an effective method for determining both the quality and quantity of RNA in gene expression analysis experiments using as little as 200 pg of total RNA - several thousand times less material than that required for gel electrophoresis. The calculation of the RQI uses an algorithm that compares three regions of an electrophoretic profile, with differential weighting, to a series of degradation RNA standards scale from 10 (intact) to 1 (fully degraded). The very simple concept behind the RQI gives results that are comparable to the RIN. The RQI is accurately calculated over a wide range of RNA concentrations (200 pg to 500 ng), is very reproducible (%CV <3), and is applicable to a wide range of mammalian tissues. The RQI number by itself is not sufficient to decide if a sample can be safely used for downstream application. The user must determine empirically what threshold their specific samples require. An upfront validation process is required to correlate RQI values and successful or failed downstream application, using a set of artificially degraded samples. This process will allow defining threshold RQI values. The Experion software groups the samples into three colour codes, which appear in a summary screen for a quick visualisation. Once the threshold values have been determined, it is possible to customize the range for each colour group. We show that quantitation of RNA using qPCR correlates well with RQI measurements. By providing an RQI score and electropherogram, the Experion automated electrophoresis system allows even the most inexperienced user to quickly and effectively quantitate the level of degradation of an RNA sample in a systematic manner in order to reliably detect differences in gene expression using RT qPCR experiments helping ensure reliability in results.

P021

Application to RAM Amplification of Real-time Analysis Methodologies Developed for PCR

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The use of model-based amplification kinetics parameter estimates may improve the accuracy and productivity of real-time amplification by extracting more information from each experiment. Algorithms implemented for PCR analysis can be usefully applied to 2-primer ramified single-stranded circle amplification (RAM.)
Although RAM and PCR kinetics are sufficiently similar to be analyzed with implementations of the same algorithms there are essential differences between the two technologies. One significant difference for kinetic analysis is that the isothermal RAM reaction can be sampled at a higher frequency than the PCR, as PCR is limited to one data point per cycle (a cycle can be sampled multiple times but those samples aim toward a single point-estimate.) By contrast, RAM kinetic data can be collected continuously (limited only by instrumentation.) Greater sampling density allows more precise identification of kinetic phase transition (e.g. baseline to exponential phase, exponential to linear phase.)
Here the application of kinetic parameter identification to RAM amplification is shown, and compared to analogous PCR analysis. While fitting parameterized models to RAM kinetics is done as for PCR, the interpretation of a RAM amplification fitted model is analogous but distinct from the interpretation of a PCR model. For example, PCR efficiency (signal increase per cycle) doubles at its theoretical maximum; in RAM the analogous interpretation is signal increase per time unit, and measures the rate of the reaction.
RAM amplification, like the PCR, can be used in high-throughput diagnostic assays. It is hoped that a more quantitative understanding of the RAM reaction will encourage broader application of the technology.

P022

Development of an alternative method for an absolute quantification of folate transporters in human tumors using bacterial artificial chromosome vector

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We have developed and validated an alternative method of the absolute quantitative real-time PCR based on the use of plasmid. Our method uses a Bacterial Artificial Chromosome vector pBeloBAC11. In contrast of plasmid, pBeloBAC11 is present in a single copy number in the bacterium E. coli EC100. Taking benefit of that we constructed a reliable standards curve based on initial input amount of BAC vector harboring a single copy of the human Reduced Folate Carrier transcript (hrfc) and the Folate Binding Protein transcript (fbp). Standard curves for each assay were highly reproducible with no significant difference in slopes between three different runs of the three different assays. The dynamic ranges were wide, ranging from 1x102 to 1x107 copies. The linearity R2 coefficient of Ct was 0.99 for the recombinant BAC. Q-PCR efficiencies were 0.991 (CV=0.09%) and 0.992 (CV=0.06%) for hrfc and fbp, respectively. The method has been applied for simultaneous quantification of the hrfc and fbp transcripts in tumor tissues and in their matched adjacent normal tissues. The method is sensitive and produces quantitative data with a good efficiency.

It may be used routinely for measuring multiple gene expression in diseases evolution.

P023

Removal of contaminating genomic DNA in QRT-PCR using a shrimp nuclease.

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DNA contamination can often occur in quantitative reverse transcription – polymerase chain reactions (QRT-PCR), and should be removed in order to avoid false positive results. DNase I is commonly used for removing DNA contamination, but this has a relatively long and harsh protocol which introduces an extra step between the isolation of RNA and the QRT-PCR reaction itself, as well as increasing the risk of RNA degradation due to the harsh inactivation conditions. A nuclease from the arctic shrimp *Pandalus borealis* , has properties that make it useful for the removal of contaminating DNA. The nuclease activity of the enzyme is specific to double stranded DNA, which therefore allows the enzyme to be added directly into the reverse transcription step. Unlike DNase I, the shrimp nuclease is easily inactivated at high temperatures, such as those used for the RT deactivation/hot start incubation step of a QRT-PCR. Here we show how the addition of shrimp nuclease during the reverse transcriptase step can remove contaminating DNA from the reaction. Human genomic DNA (100ng -10pg) was incubated with or without shrimp nuclease (10 – 0.1 units) before amplification of a 74bp fragment of the Apolipoprotein B gene was carried out. The percentage removal of genomic DNA was calculated by comparing the delta Ct values between reactions where the DNA was pre-incubated with shrimp nuclease to reactions incubated without shrimp nuclease (control). In order to determine whether the Ct shift was caused by inhibition of QPCR by shrimp nuclease, the same units of pre-inactivated shrimp nuclease was added to a separate reaction. Whilst large Ct shifts were observed at all DNA concentrations for 10-1 units of shrimp nuclease, there was also significant inhibition caused by the addition of inactivated enzyme at these concentrations. Therefore, not all of the Ct shift can be attributed to removal of contaminating DNA. However, at concentrations of between 0.8 – 0.4 units, there was no inhibition observed by inactivated shrimp nuclease and between 99.4% - 100% removal of the DNA was observed depending on the concentration. Therefore, at 0.8 – 0.4 units this enzyme effectively removes any contaminating DNA without adding an extra step to the QRT-PCR protocol - and completely eliminates the need for DNase I treatment. In so doing, it increases the accuracy and reproducibility of QRT-PCR reactions, especially when using crudely purified samples.

P024

The effect of amplicon characteristics on the success of fast QPCR.

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Adapting QPCR experiments to run using fast cycling conditions is a simple method of increasing experimental throughput by reducing run duration times by up to 50%. QPCR results and the quality thereof can be greatly affected by the characteristics of the product being amplified. This is especially true when employing fast thermal cycling protocols, which have been shown to reduce the sensitivity and increase variability of some assays. Speeding up thermal cycling protocols can lead to reaction failure if attempting to amplify a non-optimal assay or difficult target. Therefore we have investigated the effects of amplicon length, GC content and secondary structure, on the performance of fast QPCR experiments compared to when a standard thermal cycling protocol is used. A panel of human specific assays were employed in QPCR experiments using fast and standard cycling protocols. The assays were designed so that the resulting amplicons had a broad range of length, GC content and secondary structure (minimum ΔG values calculated at 60C). Experiments were carried out on the Roche LightCycler 480 and assay sensitivity was assessed by comparing delta Cp values, standard deviations and differences in QPCR efficiency (calculated by the standard curve method). The results demonstrate that amplicons that are of excessive length, high in secondary structure or high in GC content can all be causes for poor fast QPCR results. Therefore, we recommend that assays be designed with these amplicon characteristics taken into account, in order to ensure successful fast QPCR experiments.

P025

The effect of white pigmentation in 96-well plates during QPCR.

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QPCR instruments are able to monitor amplicon quantity in real-time during PCR reactions by detecting fluorescence signals and recording fluorescence data. The majority of fluorescence is reflected out of the PCR tube either by the polypropylene itself or by the walls of the thermal cycler block, when clear polypropylene is used. Aims: To investigate if white 96-well plates allow better fluorescence detection of PCR products during QPCR assays than clear 96-well plates. Methods: QPCR experiments were performed by amplifying target segments of the genes *sigB* , *dnaK* , *srfAA* , and *argB* from the genome of *Bacillus subtilis* BBK006. SYBR Green I was used to monitor product accumulation via fluorescence. To examine the effect of pigmentation on CT, 96-well plates were custom-manufactured with elevated pigment, reduced pigment or differently-compounded white polypropylene. Results: In general QPCR reactions on white plates have lower CT values compared to QPCR reactions on clear plates. Statistical analysis revealed there was no significant difference between CT values of sets *dnaK* and *argB* when using clear and white plates. However, analysis of melt curves suggested this was due to poor primer hybridisation. Further analysis revealed that CT values of sets *srfAA* and *argB* were significantly lower when using white plates compared to clear plates. There was also a significant difference between melting curves of low template DNA reactions when using clear and white plates. Conclusions: It was observed that signal noise was more prevalent in the melting curves of reactions using clear plates than white plates. There were no significant differences between CT values and amplification plots of low template DNA reactions when using the standard white plate compared to plates with altered pigmentation. This, coupled to examination of the plates by scanning fluorimetry, suggests that the effect of the pigment is complex and not necessarily linked to reflection of signal alone.

P026

Alignment of the heat shock protein gene (hsp) sequences and development of multiplex PCR method for the simultaneous detection of bovine mastitis pathogens including Staphylococcus aureus, and Streptococcus spp.

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Bovine mastitis is a multifactorial disease caused by many different bacteria species. Of these bacteria species, Staphylococcus aureus, Streptococcus agalactiae, Streptococcus uberis, and Streptococcus bovis are the major pathogens. Since the heat shock protein (hsp) genes, especially hsp60, has been shown to have more discriminatory power than 16S rRNA gene and inter-transcriptional spacer (ITS) region, in this study, we tried to develop a multiplex PCR method based on the hsp (heat shock protein) genes for the specific detection of S. aureus, S. agalactiae, S. uberis, and S. bovis. Molecular weight of the PCR products amplified were 406 bp, 350bp, 119bp, and 247bp, respectively, for S. aureus, S. agalactiae, S. uberis, and S. bovis. Using this multiplex PCR method, all the selected target strains could be specifically detected in food samples. As the multiplex PCR method was used for direct detection of mastitis pathogens in milk samples, the detection limit was N (N= 1–9) ×103 CFU/ml of milk samples. If a 10 h pre-enrichment step was performed, the detection limit was N×100 CFU/ml. Thus, the multiplex PCR method could be used for the specific and sensitive detection of these pathogenic bacteria in food and milk samples.

P027

qPCR as a method to estimate synchronization of immortal Hepa 1-6 cells – problems with inhibitors and low abundant genes

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The aim of our experiment was to synchronize the mouse immortal hepatoma cells Hepa 1-6 and to prove the success of synchronization by measuring the expression of representative genes from cholesterol synthesis and circadian regulation. These genes are known to be expressed in a circadian manner in the mouse liver. However, many of them are expressed at low levels in immortal cell lines which represents a challenge for their quantification.
Hepa 1-6 cells were grown in 12-well plates until confluency. Cells were treated with 10 uM forskolin, washed twice with ice cold PBS, lysed with TRI reagent (Invitogen) and stored at -80ºC until RNA isolation was performed according to TRI reagent protocol. Pelet was washed once with 75% etanol and resuspended in RNAse free water. Because of low RNA yield cDNA was synthesized starting from 200ng of total RNA which is a 5 times lower concentration as usual. qPCR reaction efficiency was evaluated with serial dilutions of cDNA with mCyp51 (a gene from cholesterol synthesis) and 18sRNA primers on Light Cycler 480 (Roche) using the cyber green approach. The results showed that several dilutions result in the same Cp value, while at some dilutions there was no amplification at all. After testing different serial dilutions the expected results were obtained only if cDNA was diluted to a specific (narrow range) concentration. To increase RNA amount samples with the same treatments were pooled, dried and precipitated with sodium acetate. The qPCR has been repeated with serial dilutions and the expected amplification pattern was observed (different Cp values according to dilutions).
It was concluded that the original samples contained an unidentified inhibitor of either qPCR or cDNA synthesis that has been removed by drying RNAs followed by another precipitation. After solving this problem we measured the timely expression of *Cyp51, Dbp* and *Bmal1* in a time-series experiment spanning 48 hours. In the first 8h -12h forskolin provokes an immediate early response of the 3 measured genes. According to the expression of *Dbp* that is abundant also in immortal cells, we conclude that synchronization of Hepa 1-6 cells line has been successful while the results for *Cyp51* and *Bmal1* are not conclusive. Additional optimization procedures are required to enable quantitative time-series measurements of low expressed genes in immortal cells.

P028

Automated extraction of viral RNA from blood samples for Bluetongue virus diagnosis

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Objectives - Bluetongue (BT) is a non-contagious, insect-borne disease caused by a virus from the genus Orbivirus within the family Reoviridae. The presence of viral RNA in clinical samples is monitored by silica-based nucleic acid purification followed by real-time reverse transcription PCR (RT-qPCR) analysis. This study describes a comparative validation of a manual and an automated silica-based procedure for the extraction of nucleic acids from whole blood samples.
Methodology and results - First, the linearity of the automated assay was assessed by testing a 10-fold dilution series of spiked blood with a viral load ranging from -0.32 to 5.7 log10 TCID50 ml-1. The linearity was analysed by linear regression and ranged from -0.32 to 5.7 log10 TCID50 ml-1. The limit of detection (LOD) of the automated assay was determined by analysing a 2-fold dilution series of spiked blood samples. Probit analysis was used to calculate the input concentration with a 95% probability of a positive RT-qPCR result which yielded an LOD of -1.32 log10 TCID50 ml-1. The intrarun and interrun variability of the extraction protocol is an important characteristic of the automated extraction protocol which can extract up to 184 blood samples in a single run. The intrarun and interrun variability were assessed by extracting a 1:2 dilution series of spiked blood with a viral load ranging from -1.15 to 2.46 log10 TCID50 ml-1. Each dilution was extracted automatically 5 times in each of 5 independent runs. The coefficient of variation (%) was calculated for each dilution and ranged from 1.26 to 7.26 with an increase in variation towards the LOD. A comparative analysis of both extraction protocols was made by analysing 50 field samples from the 2008 BTV epizootic that were extracted both manually and automatically. Statistical analysis using Pearson's coefficient, Bland-Altman analysis and Passing-Bablok regression analysis, all indicated that the mean difference between both procedures is negligible (-0.37) and that most results fell within the 95% confidence interval of the mean (95% CI: 0.64 to -0.10). To validate the absence of cross-contamination, 48 strongly positive and 48 negative blood samples were arranged in a checkerboard pattern. The 96 samples were extracted automatically and evaluated for cross-contamination by RT-qPCR analysis. No viral RNA could be detected in any of the 48 negative samples.
Finally, the throughput and hands-on-time of the automated extraction protocol were estimated. The automated purification of nucleic acids from 184 blood samples took 2 hours 30 minutes. Beside the important increase in capacity, the automated protocol requires much less hands-on-time resulting in a more efficient flow from sample to RNA and from RNA to qRT-PCR.
Conclusion - In conclusion, our findings clearly demonstrate that both extraction protocols give essentially the same results and that both protocols can therefore be used interchangeably.

P029

Validating internal controls for Cucurbita pepo real-time PCR studies

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Members of Cucurbitaceae family (pumpkin, squash, cucumber and watermelon) make a significant contribution to our intake of vitamins and minerals. Among them, squash (*Cucurbita pepo*) is an economically important species because of its nutritional quality, relative low price and year-round supply. Several studies have been developed in squash in order to study both quality and stress response aspects. Understanding patterns of expressed genes during squash development may provide insight into complex regulatory networks and could contribute to the breeding process of the species.
Real-time PCR is the most used method to quantify biological changes in mRNA levels. In order to control several variables in real-time PCR experiments, proper reference genes are commonly used (Vandesompele *et al.* , 2002). A need for reference genes in *C. pepo* has emerged and the studies in this area have not yet been evaluated. For this reason, we have carried out an extensive evaluation using the BestKeeper program (Pfaffl *et al.* , 2004) with the aim of studying the transcripts stability of eight commonly used housekeeping genes in thirteen *C. pepo* samples.
This work presents the details and findings from our validation of 18S, elongation factor, actin, tubulin, ubiquitin, protein phosphatase 2A, helicase and glyceraldehyse-3-phosphate dehydrogenase genes in different *C. pepo* tissues and time points (root, leaf, shoot, flower and fruit) as well as under salinity, cold and drought stresses. The normalization strategy presented here is a prerequisite to accurate real-time PCR expression profiling that opens up the possibility of developing reliable experiments in squash.

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P030

Indentification of stable reference genes for spatial and temporal gene expression studies in human brain.

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Identification of stable reference genes for spatial and temporal gene expression studies in human brain.
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Background:
Quantitative real-time RT-PCR (qRT-PCR) is a powerful tool and widely used for gene expression studies. Internal reference genes, so-called housekeeping genes, are mainly employed to normalize mRNA levels between samples. However, several studies have shown that expression of traditional housekeeping genes may vary substantially under certain conditions. Therefore, validation of stable reference gene expression is mandatory in mRNA quantitation studies using qRT-PCR. In our study, we sought to identify stably expressed reference genes in human brain tissue from several brain regions and different developmental stages.
Methods - Traditional housekeeping genes and genes recently proposed to be stably expressed under certain conditions were chosen from the literature. Expression of altogether twelve candidate reference genes were assessed in tissues from 35 different adult and 7 fetal brain regions. To calculate the most stably expressed genes and the recommended number of reference genes required for qRT-PCR normalization, data were analyzed using the software package geNorm.
Results - In the group comprising adult brain tissues from 35 different regions, the genes encoding PGK1 (phosphoglycerate kinase 1), TBP (TATA box binding protein) and SDHA (succinate dehydrogenase complex, subunit A) showed least variation in mRNA levels. In 7 fetal tissues from different brain regions, the genes SDHA, ACTB (actin, beta) and CTBP1 (C-terminal binding protein 1) were the most stably expressed genes. The genes encoding PGK1, SDHA and CTBP1 showed highest stability across the different developmental stages. Interestingly, b-2-microglobulin (B2M) and ubiquitin C (UBC), two genes frequently used to normalize qRT-PCR data, exhibited the highest variation of all tested candidate reference genes in all three brain tissue groups.
Conclusion - Employment of several housekeeping genes is needed for data normalization in qRT-PCR studies on different human brain regions and developmental stages. When studying spatial gene expression in human brain, the housekeeping genes PGK1, TBP and SDHA are recommended for normalization of expression levels in adult brain tissues, and the genes SDHA, ACTB and CTBP1 for fetal tissues, respectively. For temporal gene expression studies, the combination of PGK1, SDHA, CTBP1 and GOLGA1 (golgi autoantigen, golgin subfamily a, 1) seems to be suitable for qRT-PCR normalization.

P031

Selection of suitable reference genes for qRT-PCR for the study of circadian gene expression in mouse liver.
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Selection of appropriate reference genes is an important step in qRT-PCR that ensures accurate analysis and normalization of data generated. Circadian gene expression studies carried out by qRT-PCR have often used normalization based on one or two reference genes. 18sRNA and ß-actin are considered as housekeeping genes, though their expression has been shown to differ in certain experimental conditions, raising the question of whether they are suitable for circadian experiments. Because the number of oscillating genes is tissue dependent and because there is no guarantee that a gene oscillating in one tissue will also oscillate in the other, and *vice versa* , we tested several possible reference genes to determine the ones best suited for normalization.
A large scale circadian experiment involving 108 wild-type and 96 *Crem* knock-out mice has been conducted. Mice of similar age were housed with free access to food and water in a light-dark cycle (light: 7:00am to 7:00pm) for 14 days before they were sacrificed in dim red light every 4 h in a 24 h period. Liver samples were quickly excised, frozen in liquid-N2 and RNA was isolated from 54 wild type and 45 knock-out male mice using TRI-reagent (SIGMA). In the initial experiment cDNA from seven time points was synthesized from 1 µg of RNA and qRT-PCR reaction efficiency with serial dilutions was determined for four genes, two proposed reference genes (cyclophilin and ß-actin) and two genes under circadian control (*Cyp51* and *Npas2*). The aim was to evaluate cDNA quality and to determine the cDNA dilution to be used for subsequent high-throughput qRT-PCR.
The expression profiles of the four genes were as expected. *Npas2* and *Cyp51* showed oscillation, while cyclophilin and ß-actin were fairly constant, although there was a non-significant difference between some time points. Whether this is statistically significant needs to be proven with a larger sample number which is currently underway. The subsequent study will include the expression profile of all 99 samples from male mice for at least 5 possible reference genes and 2 known circadian genes. The application of three different selection programs (geNorm, BestKeeper and NormFinder) will aid in assessing the adequacy of selected genes to be used for normalization of the mouse liver circadian experiments.

P032

Housekeeping genes validation in acute and chronic adjuvant arthritic rat for mRNA quantification by real time RT-PCR
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Real time RT-PCR is one of the efficient methods in mRNA quantification. One of the approaches for quantification is the normalization of mRNA by internal control gene or housekeeping gene. The expression level of internal control should remain constant in different tissue types and in different experimental conditions for efficient quantification. In the present study we have compared the expression levels of 10 commonly used housekeeping genes in 9 different tissue types in normal, acute and chronic adjuvant arthritic conditions in rat. We have also studied CD3 gene expression in ankle joint and cortical bone to evaluate the differences in the levels of housekeeping gene expressions due to inflammatory cells. Our data showed an increased of all HKGs in acute and chronic adjuvant arthritis. HPRT was the most stable housekeeping gene among all the tested ones in acute and chronic adjuvant arthritic conditions in most of the tissue types in rat except for the ankle joint and cortical bone. There was no stable HKG in both the tissues in acute and chronic condition. We have also found a significant increase in CD3 gene expression which was significantly correlated with the increased expression level in ankle joint and cortical bone in both acute and chronic adjuvant arthritic rats. In conclusion our data suggested HPRT to be the most stable housekeeping gene in most of the tissue types in adjuvant arthritic condition in rat. Further more an increase in the level of housekeeping gene expression in ankle joint and cortical bone is due to the increase of inflammatory signatures in acute as well as in chronic adjuvant arthritic rats

P033

Comparison of high and low virulence serotypes of Actinobacillus pleuropneumoniae by quantitative real-time PCR
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Until now, 15 different serotypes of *Actinobacillus pleuropneumoniae* (Ap) have been described based upon differences in the capsular polysaccharides of the bacterium. The virulence of different serotypes of Ap has been experimentally determined and the differences in mortality and morbidity are considerable. The genetic mechanisms behind these variations in virulence are largely unknown, and for bacteria in general, the non-virulent strains often contain many of the virulence genes required for an infection. In Denmark, serotype 2 and serotype 6 are the most commonly found, with serotype 2 being of high virulence while serotype 6 strains are normally found to be less pathogenic. To gain an understanding of the differential virulence of serotype 2 and 6, the expression of a panel of Ap genes during infection of porcine epithelial lung cells (SJPL) were examined by quantitative real-time PCR (qPCR). Flasks of SJPL cells were inoculated with equal amounts of Ap serotype 2 and 6, respectively. After two hours, the supernatant was discarded, the cells and attached bacteria harvested, and total RNA isolated. After an enrichment

step for bacterial RNA, the expression of a number of Ap genes believed to be important for early establishment of the bacteria in the host were examined by qPCR. Three previously validated reference genes, *glyA* , *pykA* and *tpiA* were included for normalization of the qPCR data. Among the eight genes compared, three appeared to be significantly differently expressed. In serotype 6, the genes *cpxB* and *kdsB* , both involved in capsule formation, were upregulated compared to serotype 2. In serotype 2, the production of *pgaB* , involved in biofilm formation, seem negligible in comparison to serotype 6. This result is in agreement with observations in the laboratory, where biofilm formation is observed for some strains of serotype 6. Interestingly, in serotype 2, the toxin gene *apxIIA* appears to be significantly upregulated compared to serotype 6, which is in accordance with the observed difference in pathogenicity. However, further investigations will be needed to establish whether these results have any relevance to the difference in virulence. Results from this study will be used as the basis for a microarray approach to examine the overall gene expression variation between Ap serotypes in vitro and in vivo.

P034

The effect of nucleic acid extracts on the reverse transcription step

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Reverse transcription quantitative PCR (RT-qPCR) is an established and accurate method for measuring RNA from clinical samples. The reverse transcription (RT) is described as being the most variable step of RT-qPCR analysis, yet can be overlooked when considering optimisation and quality control. The effect of sample inhibition upon the qPCR step is well recognised, with the risk of producing underestimated measurements or even false negative results. However, there is little information or discussion regarding the same effect on the RT step. In this study we specifically investigate potential inhibition of the RT reaction by examining the impact of clinical nucleic acid extracts on the result. In a model of infective endocarditis RT reactions were performed on bacterial RNA in the presence of different concentrations of nucleic acid extract from human heart valve material. The effect of the heart valve extracts on both the RT and PCR steps were then assessed by comparing to an uninhibited control reaction.
Our findings demonstrate that human heart valve extracts can considerably inhibit the RT reaction. While samples that inhibited the RT step were also more likely to inhibit the qPCR step the former was automatically at more risk due to the stepwise nature of the protocol (i.e. the extract always being more concentrated in the RT than the subsequent qPCR). Furthermore we demonstrate that some extracts enhance the RT reaction. It is clear from our findings that components co-purified during the RNA extraction can have considerable positive or negative effects on the RT step. This has implications for all RT steps and researchers should be aware of the potential important inaccuracies that may occur due to this previously undescribed phenomenon.

P035

Comparison of two available platforms for the determination of RNA quality

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The integrity of RNA is a very critical aspect regarding downstream RNA based analysis. Low-quality RNA can compromise the results of such experiments. To save time, costs and material, platforms for the determination of RNA quality play an important role. Two available systems for the determination of RNA quality are the Experion (Bio-Rad) and the Bioanalyzer 2100 (Agilent Technologies). Both platforms determine RNA quality either by using the ribosomal 28S /18S ratio, or a numerical system which represents the integrity of RNA. The Bioanalyzer offers the RIN algorithm (RNA Integrity Number) on the Bioanalyzer 2100 and Bio-Rad developed a new Experion software version that offers an algorithm for calculating the RNA Quality Index (RQI). The aim of these experiments was to compare both systems regarding sensitivity, reproducibility and the influence of individual tissue extractions and different runs. Therefore RNA quality of 6 different bovine tissues was determined in six extraction replicates. Moreover the connection between measurement sensitivity and RNA quality should be shown by 6-11 dilution step series with artificially degraded RNA. Every chip composition was simultaneously measured in the Experion and the Bioanalyzer 2100. The experiments show that results obtained from both systems are significantly different between the extractions applied, but not influenced by multiple runs and chips. Regarding reproducibility and absolute sensitivity, it could be demonstrated, that data obtained by measurements on the Experion show slightly better results. In the lower RNA quality areas (RIN/RQI 3 to 5) the Bioanalyzer 2100 shows a higher linearity. Overall it was shown that both algorithms are very comparable and beneficial for the determination of RNA quality for downstream applications, like qRT-PCR or hybridization arrays.

P036

Evaluation of different extraction protocols of total RNA and microRNA from bovine blood for gene expression analysis

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Blood is the most important tissue source for molecular diagnostics because it is most easily accessible. Quantitative real-time RT-PCR (qRt-PCR) has improved the efficiency of gene expression analysis. But sampling, handling and the extraction procedure can highly impact variability of gene expression results. This led us to compare five different blood RNA sampling and extraction protocols to find out which guarantee the best outcome with respect to highest yield of RNA and microRNA concentration and lowest crossing point (CP) with least variance in qRT-PCR applications. Blood samples were collected in triplicate from 5 healthy Brown Swiss heifers. Two phenol based extraction methods [Trifast], namely direct extraction of whole blood (WB) and leucocyte extraction after lysis of erythrocytes (LY) were designed to isolate total RNA including mRNA and microRNA in one fraction. PAXgene [Qiagen] (PAX) and LeukoLOCK [Applied Biosystems] (LL) sampling devices as well as the extraction of the plasma interphase of coagulated centrifuged blood (PI) were performed to separately isolate mRNA (>200nt) and small RNA (<200nt) [Qiagen]. RNA and microRNA were extracted from either whole blood (WB and PAX) or blood leukocytes (LY, LL and PI). For each extraction protocol RNA yield was quantified [Nanodrop], quality and integrity was obtained [BioAnalyzer] and gene expression was measured by qRT-PCR [Eppendorf] of 11 mRNA and 7 microRNA target genes. The best results in terms of highest quantity, quality and best performance for mRNA qRT-PCR were obtained for the samples extracted by the LY and PAX methods. Moreover there is a tendency to obtain the best microRNA results for the samples where total RNA was extracted in one fraction (LY and WB). The PI method show the worst results due to high variance in all variables considered in this study. Since normalization could reduce the effect of RNA integrity on gene expression the extraction of whole blood or leucocytes showed minor differences. In summary, the results obtained offer an overview of practical concerns of different mRNA and microRNA extraction methods from bovine blood. As superior quality parameters where obtained with different extraction protocols, the method of choice has to be based on the necessity of the study conditions.

P037

Application of qPCR method for transgene copy number determination in recombinant CHO cells in biopharmaceutical industry

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The majority of human therapeutic proteins are favorable to be produced in mammalian cell-culture systems due to their ability of proper folding, postranslatic modifications and glycosylation profiles of therapeutic proteins. Since determination of transgene

copy number and genetic stability are among the important parameters for recombinant protein production, the focus of this study was the application of qPCR method for this purpose. In presented work Chinese hamster ovary (CHO) dhfr- cell line was used for expression of the recombinant protein underwent antibiotic and methotrexate selection. A method for gene copy number determination using absolute quantification and TaqMan chemistry was developed. Specific primers and probes for transgenes of A and B subunit of recombinant protein and endogenous gene on CHO chromosomal DNA were constructed. A ratio of copy number between recombinant and endogenous gene was calculated and presented as a transgene copy number per diploid genome of CHO cell. The method was successfully applied for genetic characterization and stability study during development and final characterization of cell line for recombinant protein production. In addition some interesting findings were observed, a correlation between gene copy number and specific productivity after amplification with methotrexate as well as a correlation between the amount of plasmids containing recombinant and resistance gene respectively, used for cotransfection in comparison to the transgene copy number.

P038

Comparison of two different types of preamplification

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Accurate gene expression quantification by qRT-PCR could be limited by a low RNA quantity obtained from clinical samples (laser microdissection, circulating tumor cells or FFPE tumor tissue). One of the method that helps to overcome the small amount of the sample material and to lower the originally high qRT-PCR CTs is preamplification of cDNA. Here, we present the comparison of two types of preamplification, preamplification of cDNA using the TransPlex Whole Trascriptome Amplification Kit and gene specific preamplification. We compared expression profile of 40 genes (breast cancer markers and housekeeping genes) in FPPE tumor tissue. We used Biomark instrumentation for qRT-PCR. The molecular profile of the samples was analyzed by biostatistical method.

P039

Effects of formalin, methacarnoy and FineFIX fixation on RNA recovery and preservation

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Archival formalin-fixed and paraffin embedded tissues represent the most abundant supply of clinical material on which molecular analyses can be now performed for translational studies. Formalin, however, has detrimental effects on nucleic acids and proteins, impairing their extraction efficiency and suitability. Moreover, formalin represents a hazardous chemical component. In recent years several alternative fixatives have been introduced in order to overcome these limitations.
In the present work, formalin was compared with two alcohol-based fixatives, methacarnoy and the non toxic commercial FineFIX, with respect to their effect on RNA recovery and preservation. The effect of time of fixation (from 1 to 24 hours) was evaluated in a cell line-based model. The effects of fixatives were then investigated in a panel of human tissue samples to evaluate the impact of further variables (e.g. time of fixative penetration, tissue composition) on RNA impairment. Fresh cells and tissues were used as control. Total RNA yield was measured by Nanodrop, rRNA integrity was analysed by Agilent Bioanalyzer, mRNA integrity by endpoint RT-PCR amplification of fragments of increasing length and mRNA quantity by realtime RT-PCR.
In the cell line model RNA recovery as well as rRNA and mRNA preservation were affected by all the fixation procedures, although to a different extent. Formalin fixation showed a time-dependent detrimental effect on both total RNA recovery, rRNA/mRNA integrity and mRNA quantity. Methacarnoy and FineFIX resulted more conservative than formalin on RNA yield, mRNA integrity and quantity and their effect was time-independent. However, RNA from methacarnoy-fixed samples showed a better rRNA preservation with respect to that from FineFIX-fixed specimens. In tissues, high rRNA degradation levels were noticed in all the differently fixed specimens, while the detrimental effects of formalin and the protective effect of both alcohol-based fixatives on mRNA integrity were all confirmed. Accordingly, in methacarnoy-fixed samples also mRNA quantity was preserved, resulting comparable to that obtained in controls. Conversely, in FineFIX-fixed samples mRNA expression results were quantitatively similar to those obtained with formalin treatment.
In conclusion, we showed that both aldehyde- and alcohol-based fixatives have detrimental effects on overall RNA preservation but the entity of their effects is fixative-specific and is dependent on variables related to the use of tissue samples. In particular, the time-independent effects of alcohol-based fixatives make them practical for RNA investigations even in specimens fixed for very long periods. Moreover, in our hands methacarnoy results the most suitable fixative for RNA analyses in small clinical samples because it ensures the highest level of RNA recovery and preservation.

P040

Real time monitoring of cell behavior on the xCELLigence system supports qPCR analysis of dynamic gene expression

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The analysis of gene expression patterns after candidate drug treatment of cell lines is a crucial step in understanding drug activity and in the identification of biomarkers which supports the identification of patients who will benefit from a new drug. Here we describe a model system in which we combine the online monitoring of cellular events after drug treatment using the xCELLigence system with qPCR analyses using RealTime ready panels. Cell growth curves recorded by the xCELLigence system after treatment with candidate drugs for the first time provide means to identify optimal time points for sample collection for subsequent RT-qPCR analysis. Here we treated the colon cancer cell line HT29 with a previously defined effective concentration of the anti-cancer drug paclitaxel. The growth behavior of the treated cells was monitored in comparison to the growth behavior of control cells. The expression level of 84 apoptosis-related genes was compared among treated and untreated samples collected at different time points using the Real Time ready Human Apoptosis Panel, 96 on LightCycler®480 Instrument. For data analysis the LightCycler®480 Multiple Plate Analysis Software was used. The results show that xCELLigence supports the knowledge based decision for ideal time points in the analysis of dynamic gene expression patterns by RT-qPCR.

P041

STABILITY OF ENDOGENOUS CONTROL GENES IN HUMAN POST-MORTEM TISSUE

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The possibility to use human tissue obtained during forensic autopsy for extraction of RNA in suitable quantity and quality for gene expression analysis was shown previously. Nevertheless it is undoubted that special care should be taken when working with human tissue samples from individuals with long postmortem intervals which are common in forensic routine work. A correct data normalisation strategy that is able to eliminate effects from changing postmortem conditions as well as various causes of death is indispensable before performing gene expression studies.
In our study we examined the stability of 10 potential endogenous control genes in different human postmortem tissues by calculating an average expression stability value. The stability of these genes will be presented with respect to different causes of death and varying postmortem intervals. The minimal number of endogenous control genes needed for correct data normalisation in gene expression studies in human postmortem tissue as well as appropriate candidate control genes will be presented.

P042

SYTO9-based real-time PCR and electron microscopy for quantification of colloidal gold-bound oligonucleotides and antibodies

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Colloidal gold nanoparticles (GNPs) armed with oligonucleotides alone or together with antibodies are increasingly used for sensitive detection of DNA or proteins. An important step for optimizing the sensitivity and reproducibility of GNP-based assays is the quantification of oligonucleotides bound to the particles. To this end, several techniques including real-time PCR with fluorescent beacon probes have been described. However, these techniques are either insensitive or require expensive probes. Here we report a SYTO9-based real-time PCR method for quantification of oligonucleotides bound to GNPs. Thiol-capped oligonucleotides were attached to 30 nm GNPs (30GNPs). Unbound oligonucleotides were removed by centrifugation of the particles through 30% glycerol. Pelleted particles were then dispersed in phosphate buffer (pH 7.4) with 0.15 M NaCl and 1% bovine serum albumin (BSA). In some experiments, rabbit polyclonal antibody specific for selected antigen was bound to 30GNPs, followed by binding of thiol-capped oligonucleotides. To detect gold-bound oligonucleotides, the particles were pelleted and resuspended directly in 1x PCR master mix containing SYTO9 fluorescent dye, TaqDNA polymerase, dNTPs, buffer, short template DNA complementary to the 3' end of the oligonucleotide bound to 30GNPs, and two unlabeled primers complementary to the 5´ end of the gold-bound oligonucleotide and 3' end of the template. Routinely, 40 cycles of PCR were monitored using real-time Mastercycler Realplex (Eppendorf). No inhibitory effect of 30GNPs was detected on SYTO9-based real-time PCR. An indirect relationship was evident between the amount of oligonucleotides bound to 30GNPs and the Ct values obtained. Calibration curves were constructed using free thiol-capped oligonucleotides. Rabbit antibodies bound to 30GNPs were detected with commercial 5 nm GNPs armed with goat-anti rabbit IgG (GaR_5GNPs). Briefly, armed 30GNPs were bound to an electron microscopy grid covered with pioloform film and poly-L-lysine. After washing and blocking with BSA, GAR_5NPs were added. Unbound particles were removed by washing and dry samples were observed by electron microscope. When properly prepared, 30GNPs with bound rabbit antibodies were surrounded by GaR_5GNPs forming rosette-like structures. Usually, >90% antibody-armed 30GNPs formed rosettes. When 30GNPs were armed with unrelated protein, almost no GAR_5GNPs were bound, confirming the specificity of the assay. The results indicate that SYTO9-based real-time PCR offers a rapid, simple and inexpensive method for mapping the surface coverage of oligonucleotides bound to GNPs. In combination with detection of antibodies bound to the same particles it is possible to monitor properties of nanogold conjugates. Such data are extremely important in standardization of bio-barcode amplifications and other assays used for detection of proteins. Supported by KAN200520701.

P043

The tilted threshold line. An alternative to the horizontal counterpart which is commonly used for dilution curve quantification?

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The estimation of real-time efficiencies has been shown to be conducted most accurately by employing calibration curve analysis. For this purpose, serial dilutions of the gene of interest are subjected to real-time PCR and the threshold cycles obtained from the dilutions are plotted against the logarithmized dilution factors (relative approach) or copy numbers (absolute approach). The threshold cycles (or otherwise denoted as crossing points) for the above procedure are usually acquired from the software accompanying the real-time PCR instrument. This value is instrument specific and may often be not be very intuitive for the user.
A major drawback is to our opinion, that the threshold cycles are always calculated from a fluorescence value which is constant over all dilutions and samples (threshold line or border). This results in suboptimal regression curves when the difference in the threshold cycles (delta-ct's) between two dilutions gets larger with increasing dilutions, which is almost always seen for higher cycles. Being able to accurately quantify samples with very low abundance of transcripts is crucial, as these often belong to the most interesting biological groups (i.e. transcription factors or receptors).
We developed an approach which might tackle this problem. Our method subtitutes the horizontal threshold line by a a line which is tilted downwards from the sample of lowest dilution to the sample of highest dilution. This line with slope X and intercept Y is calculated iteratively through all intercepts and slopes (calculation time about 2-10 minutes, depending on the number of steps chosen within each iteration), and ultimately a threshold line with defined X and Y is selected that optimizes the performance of a regression curve, either by maximizing the coefficient of determination R-square or by minimizing the Akaike Information Criterion (AIC). Constraints are applied to this curve such that the iteration starts at the second derivative maximum of the lowest dilution sample and stops at the first outlier cycle of the highest dilution curve, so that values in the baseline region of the curves are not accidentally included. We show that with this method, a gain in accuracy and precision for those regimes can be achieved, where there is a need in quantitation of very low abundance genes.

P044

Establishment of robust normalizers for the qRT-PCR analysis of kidney and heart under diabetic conditions and upon the treatment with the copper(II) chelator TETA in R. norvegicus

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Objective: Diabetic nephropathy and diabetic cardiomyopathy are severe diabetic complications. An experimental treatment for the diabetic cardiomyopathy and the diabetic nephropathy is a normalization of the copper homeostasis in these organs using the Cu-selective transition metal chelator trientine (TETA) (Cooper *et al* ., 2004). We aim to better understand the molecular mechanisms by which TETA treatment leads to the reverse of diabetic heart and kidney function in the animal model of STZ-induced diabetic rats. Here, we report the establishment of robust normalizers suitable for qRT-PCR assays of diabetic heart and kidney and show the importance of proper normalization in the context of the expression analysis of genes of glutathione metabolism.
Methodology: Animal experiments using STZ-rats were performed according to ethical standards. Total RNA was extracted from left ventricle of hearts and renal cortex, followed by cDNA synthesis. cDNA concentrations were determined and equal amounts of cDNA were used in each qPCR run. Standard and melting curves were performed for every gene in every run. An efficiency corrected ΔCt method was used for calculation of relative expression levels. Candidate reference genes were analyzed via the NormFinder (Andersen *et al* ., 2004) and the geNorm (Vandesompele *et al* ., 2002) algorithms. The relative expression levels of our genes of interest were calculated according to Pfaffl *et al* . (2004).
Results: We analyzed U2AF, NDC1, RPL13a, PIPA, YWHAZ, TBP, GAPDH, bACT and 18S as candidate reference genes. Total cDNA was also analyzed as a possible normalizer. U2AF, TBP and bACT were rated the best candidate reference genes in kidney according to both algorithms. For the heart both algorithms rated NDC1, TBP and RPL13a as the most stably expressed candidate reference genes.
For the expression of Glutathione S-transferase kappa 1 we show that usage of a 'bad' normalizer can reverse the trend observed with a proper normalizer.
Conclusions: We have identified sets of reference genes to build robust normalizers for qRT-PCR analysis of kidney and heart under diabetic conditions and upon the treatment with TETA. We further show for the first time that U2AF and NDC1 are reasonable candidate reference genes, expanding normalization to genes coding for spliceosomal components and parts of the nuclear pore complex.
Abbreviations: TBP: TATA-box binding protein, NDC1: Nucleoporin1, U2AF: U2 snRNP auxiliary factor, bACT: β-Actin, GAPDH: Glyceraldehyde-3-

phosphate dehydrogenase, PIPA: Peptidylprolyl isomerase A, YWHAZ: Tyrosine 3-monooxygenase, 18S: 18S rRNA, RPL13a: Ribosomal protein L 13a

References: Cooper et al. (2004) Diabetes 53:2501-2508, Andersen et al. (2004) Cancer Research 64(15):5245-50, Vandesompele et al. (2002) Genome Biology 3: 0034.1-0034.11, Pfaffl, et al. (2004) Biotechnol Lett 26(6):509-515

P045

How to validate qPCR method for transgene copy number determination in recombinant CHO cell line to be in compliance with GMP (good manufacturing practice) in biopharmaceutical industry?

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In biopharmaceutical industry stringent requirements take place for validation of analytical methods used for characterisation of products and cell lines producing them. Genetic characterization of cell lines chosen for production, from which MCB (master cell bank) and WCB (working cell bank) are prepared, is a first step under the cover of GMP in a process of recombinant protein production development. In addition to prove sequence by sequencing, control length and differentiate integration sites of transgene by Southern blot analysis and check length of transcript by Northern blot analysis, a qPCR method for transgene copy number determination was introduced. For this purpose the qPCR based method had to be validated in a proper way according to the guidelines. Particular assays were designed to prove the accuracy, precision (intra-assay, intermediate), specificity, selectivity, linearity, determine limit of detection, limit of quantification, range of quantification and test ruggedness and robustness of the developed method. On a basis of data collection during method qualification acceptance criteria for above listed assays were set. In this work, case study, challenges, difficulties not easy to overcome and solutions arisen during the design of assays and testing of parameters are addressed and presented. The outcome of our work is validation protocol, which is used in our laboratory to verify qPCR method for transgene copy number determination and assure reliability of the obtained results.

P046

RealTime ready – Functionally Tested qPCR Assays based on the Universal ProbeLibrary for Gene Expression Analysis on the LightCycler® Platform

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With the new introduced RealTime ready Focus Panels, Roche Applied Science now offers a convenient and reliable solution of content supply specifically for the LightCycler® 480 System. The RealTime ready assays are based on the unique Universal ProbeLibrary technology which allows fast and flexible assay design for millions of targets from virtually any organism for Gene Expression Profiling applications. The Universal ProbeLibrary consist of just 165 short probes providing transcriptome-wide coverage in most organisms. These short probes are highly specific and possess the high melting temperature (Tm) required for real-time PCR due to the incorporation of locked nucleic acid (LNA).
Each RealTime ready qPCR assay is extensively validated covering sensitivity, PCR efficiency, specificity and signal intensity. The RealTime ready assays are dried-down in micro well plates and currently available as content panels (specific for certain cellular pathways, protein or gene families etc). The relevant genes for certain pathways and gene families were selected in cooperation with experts and are now available as pre-plated, ready-to-use assays.
Full customization of RealTime ready is planned for 2009. This will enable researchers to create their own panels on an easy-to-use online configuration portal or to order single assays for their targets of choice.
The presented results demonstrate that the RealTime ready Focus panels show very high performance in real-time PCR based expression assays compared to other technologies. These new tools offer major advantages like the combination of high flexibility and high specificity and additionally a reduction in time from assay design to experiment. Thus, the RealTime ready Focus panels provide a robust expression profiling platform highly useful for high-throughput expression analysis, especially for microarray validation and gene knock-down experiments.

P047

Mechanism of mRNA degradation in Xenopus laevis

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Cell determination during early development depends on mRNA and protein expression and the distribution of the molecules within and among cells. Also mRNA degradation plays an important role in early development. Production and degradation of mRNAs must be precisely controlled for normal development of an organism. Our aim is to study mRNA degradation mechanisms during early development of the African clawed frog Xenopus laevis that occur post mortem. We applied real-time RT-PCR and a new 5' / 3' approach. Our results suggest that post mortem mRNA degradation is rate limited by the initiation of the degradation process. Once this starts the entire mRNA is rapidly degraded.
This work is supported by GAČR 301-09-1752 and GAAV IA500970904

P048

Easy and Reliable qRT-PCR Analysis of Total RNA Isolated from Fresh Frozen and FFPE Tissue Samples

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The use of tissue sections for molecular analysis of pathogenic states in mammalian tissue has become an indispensable approach for the understanding of molecular mechanism in etiology and disease progression. In particular, tumor genesis, as a very local process, requires individual sample preparation and conservation. Most commonly, dissected tissue samples are prepared as fresh frozen or even more often as Formalin-Fixed Paraffin Embedded (FFPE) samples. Both techniques have advantages and disadvantages. The most severe drawback of FFPE samples is the degradation of RNA during the sample conservation. Although fresh frozen sample material better reflects the pathological state of a tissue, the challenge to economically organize the required logistical chain - necessary for fresh frozen tissue samples - have not been satisfactorily solved. Here we present data for two independent workflows for gene expression analysis using Roche's LightCycler®480 Instrument with the SYBR Green I and the UPL format. RNA samples were isolated from FFPE or fresh frozen HeLa xenograft tissue. Either the manual method using the High Pure FFPE RNA Micro Kit or the automated method using the MagNA Pure LC RNA Isolation Kit III (Tissue) on the MPLC 2.0 Instrument was employed. Isolated RNA was subjected to cDNA synthesis using the Transcriptor First Strand cDNA Synthesis Kit. The results show that a robust workflow leading to excellent data sets suitable for gene expression analysis can be established by combining Roche products.

P049

Fast blocks and SsoFast reagents; Comparison of next generation instrumentation and reagents to their predecessors.

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Reducing run times for PCR and real-time PCR assays can dramatically increase throughout and accelerate scientific discovery. Next generation instruments and reagents open the door to generating rapid and robust assays, but steps must be taken to ensure compressing run times is not detrimental to the sensitivity and to the reproducibility of low expression targets. With Bio-Rad's next generation of real-time PCR supermixes, fast real-time PCR can be achieved without sacrificing assay reproducibility or sensitivity. We present strategies and techniques to reduce PCR and real-time PCR assay times without compromising the quality of results.

microRNA – siRNA Applications
Location: poster room (mensa)

P050

Molecular phenotyping of miRNA perturbation in neuroblastoma

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miRNAs are an emerging class of small non-coding RNAs involved in the regulation of oncogenes, tumor suppressors, and a number of cancer-related genes. The potential of miRNAs as therapeutic molecules and targets in cancer pathogenesis has increased the urgency of studying miRNAs in the field of cancer research. This abstract presents an innovative study, in which functional screening of both the whole human Pre-miR™ and Anti-miR™ libraries is performed on different neuroblastoma cell lines, followed by the expression profiling of various genes involved in cancer-related pathways.
Neuroblastoma (NB) is the most common extracranial solid tumor of childhood and comprises of about 15% of all childhood cancer deaths. The whole human miRNA assay panel was first used to profile miRNAs involved in MYCN-amplified neuroblastomas. Several miRNA were shown to be fundamental components of the MYCN transcriptional network that mediate the MYCN tumorigenic program. Transfection of NB cell lines with miRNA inhibitors and precursors directed against the up- and down-regulated miRNAs respectively resulted in a profound decrease of cell viability indicative for oncogenic and tumour suppressor activity.
Functional screening of 470 Pre-miR™ and 470 Anti-miR™ will be performed next on 10 neuroblastoma cell lines. Some of these cell lines are used as control, others have interesting aberrations in genes known to play a role as oncogene or tumor suppressor (such as MYCN amplification). After transfection, the cells are lysed with Cells-to-Ct and expression profiling is done by RT-qPCR on a panel of 20 genes involved in cell cycle, apoptosis, cell migration, and angiogenesis.
The first findings indicate that miRNAs are fundamental components of the MYCN tumorigenic program. The whole Anti-miR™ and Pre-miR™ library screening is an innovative way to discover which miRNAs are involved in the regulation of important pathways related to cancer pathogenesis and thus potential new therapeutic molecules in cancer treatment.

P051

Isolation of small RNA species from PAXgene Blood Tubes

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Sampling of blood is a standard procedure in most clinical trials and many diagnostic testing procedures. Pathological conditions in organs and remote tissues are often detectable in gene expression profiles from blood samples. Artificial modifications of the RNA content and profile of a given blood sample between collection and isolation procedure caused by degradation and gene induction are well documented (Rainen et al. 2002 Clin Chem 48: 1883-90; Müller et al. 2002 Leukemia 16: 2395- 99). Doubtful results are the consequence especially for very sensitive analysis methods like qRT-PCR assays and gene chip experiments. So the need for stabilisation of cellular RNA species to freeze the gene expression profile at the time of blood collection is widely accepted and the PAXgene Blood RNA stabilisation and isolation systems are commonly used to address this problem.
Since the standard PAXgene Blood RNA protocols were designed for mRNA purification the isolation of miRNA from PAXgene Blood RNA tubes is not very efficient, as the binding conditions are not optimized for small RNA species. A first attempt to optimize these conditions and to allow a more efficient purification of small RNAs from PAXgene Blood RNA tubes was published recently (Kruhøffer et al. 2007 Vol 4(4): 452-8). Due to several drawbacks of this method, we developed a new protocol and chemistry to achieve optimal yields of small RNAs from PAXgene Blood RNA Tubes.
Yield and quality of small RNA species were determined on the Nano and small RNA Labchip using an Agilent® 2100 bioanalyzer as well as with classical gel analysis. Purified RNA was analyzed for genomic DNA contamination and the presence of PCR inhibitors. As a downstream method different quantitative RT-PCR assays for miRNAs based on SYBR green or probes were used.
We could show a clear enrichment of small RNAs by gel analysis and on different Agilent Bioanalyser chips for manual as well as for automated miRNA protocols compared to the corresponding standard PAXgene protocols. High miRNA contents could be confirmed with different qRT-PCR assays detecting miR-10a, 16, 30b, 192 and let7a. Very low amounts of gDNA (< 1%) were present in the eluates and they showed no inhibition of RT-PCR. We saw no interference with qRT-PCRs when detecting different mRNA transcripts.
These results show that the dedicated isolation procedures for small RNAs based on the PAXgene Blood RNA Tube result in high quality enrichment of these RNA species which are ready for direct use in sensitive downstream applications.
Disclaimer: PAXgene Blood miRNA system is intended for Research Use Only. Not for use in diagnostic procedures.

P052

Quality control of miRNA in biological extractions

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In recent years, hundreds of miRNAs have been discovered, but their functional roles remain to be classified in detail. The expression level of individual miRNAs is typically quantified by methods like qRT-PCR or microarray hybridization. One of the major drawbacks in miRNA research is the lack of adequate analytical methods for small RNA extractions before going in the subsequent analysis of miRNA fractions.
Here we present a highly sensitive microfluidic assay for the analysis of small nucleic acids. The assay allows detecting miRNA fraction down to a concentration of 50 pg. It measures integrity, size and concentration of small RNA and is especially calibrated for miRNA. Those features allow researchers to optimize small RNA isolation and purification protocols, to monitor the ratio of miRNA fractions in total RNA extracts, and to screen siRNA preparations. In conclusion, our approach allows the implementation of miRNA quality control in an expression workflow by monitoring miRNA fractions derived from different tissues or extraction methods.

P053

Micro RNA Gene Expression Profiling of Colorectal Cell Lines: Prediction of Drug Sensitivity and Correlation to Mutation Status

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Gene expression profiling of 56 colorectal cell lines by qRT-PCR was performed against a panel of 384 miRNAs using Low Taqman Density Arrays (TLDAs, Applied Biosystems). Cell lines were predominantly isolated from patients with Dukes stage B-D disease and there are two lines from stage A (SW1116 & C106). Data analyses of drug sensitivity for the colorectal cell lines have identified: 13 significantly, differentially expressed miRNAs common to two oncology compounds that are EGFR inhibitors and 10 miRNAs significantly, differentially expressed miRNAs common to two oncology compounds that are MEK inhibitors. These miRNAs are potentially predictive of drug sensitivity in *in vitro* models of colorectal cancer.
Additionally, the linkage between mutation status for BRAF, KRAS and p53 and miRNA gene expression of the colorectal cell lines was tested. Statistical analyses were performed on a subset of 177 miRNAs for the following groups: BRAF (37 wild type & 10 mutant), KRAS (20 wild type & 25 mutation) and p53 (10 wild type & 34 mutation). Permutation testing revealed that the most significant miRNA gene expression change was with p53 mutation status. 20 miRNAs with highly significant, differential gene expression ($p < 0.05$) have been identified and correlated to p53 mutation status. Significant differential miRNA gene expression linkage for BRAF and KRAS in this colorectal cell line panel was not demonstrated.
This study has validated the potential of miRNA gene expression as a powerful tool to identify biomarkers for the prediction of drug sensitivity and linkage to mutation status in colorectal cell models.

P054

qPCR applications for Drug Discovery
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In the CVGI (Cardiovascular and Gastro-intestinal) department at Astrazeneca our work focuses primarily on diabetes and obesity research. We routinely use the QPCR technique throughout all stages of the drug discovery process from target validation through to lead optimisation, for tissue expression profiling, assessing the effects of treatments and diet on gene expression, verifying knockdown of a gene by siRNA and assessing expression of a transgene in transgenic mice. We run our qPCR on the ABI 7900 platform, which allows us to utilise 96 well, 384 well and microfluidic card formats. For larger studies we use an automated system from Qiagen, the Qiacube, to prepare RNA from our homogenised samples and are currently looking into automated systems to optimise the other steps in the process.

P055

Next generation siRNAs: combining innovative design with novel chemical modifications, for superior consistency of phenotypic results.
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Poor efficacy of siRNA prediction and poor specificity too often combine to wreak havoc for RNAi users. To alleviate these problems, we used classification bioinformatics and novel nucleic acid chemistry to produce the next generation of siRNA technology resulting in improved coherence of phenotypes between siRNAs to the same mRNA and higher siRNA potency. We employed a support vector machines (SVM) strategy to predict highest potency siRNA. Maximum silencing was obtained with 90% of the siRNA predicted using this algorithm at doses 5-100x lower than all other currently commercially available siRNAs. In addition we compared LNA® and 2'OMe in strategic placements of modifications throughout the siRNA structure and found a novel pattern of LNA® placement that improves siRNA specificity without disrupting the potency of the siRNA. The top ranking siRNAs predicted by the algorithm produce consistent maximum knock-down and the lead modification gave the smallest footprint in microarray experiments compared to alternative chemistries or unmodified siRNA with the same sequence. High-content microscopy testing of a custom library of genes demonstrated that >90% of the siRNAs tested elicit the expected phenotype, showed reduced off-target cell effects compared to unmodified siRNA and produced coherent phenotypes between siRNAs. The SVM strategy, results of the chemical modification screen and RNAi performance supporting data will presented.

P056

The application of RNAi for studying of isoflavone synthase gene family in red clover
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Isoflavones are mostly restricted to the Fabaceae family. Within Fabaceae family, red clover has recently received considerable interest as a valuable source of isoflavones. It has been suggested from the previous studies that isoflavones play an important role in plant defense system in response to the number of stresses including attack by pathogens, UV light, and physical and chemical damage. However, it is supposed more concentration of isoflavones increase red clover responses to biotic and biotic stress. In this study, RNA interference technique was used to study role of IFS gene family, a key gene in isoflavone biosynthesis. Inverted repeat binary vector of IFS gene has been made using Gatway technology and transferred via Agrobacterium-mediated system to red clover. Using q-RT PCR we showed dramatically decrease in IFS gene in comparison to control plants. The transgenic plants will be investigated by more molecular techniques. Key words: qRT-PCR (quantitative real-time polymerase chain reaction), RNA interference, Gatway technology, red clover Abbreviations: qRT-PCR, reverse transcription followed by quantitative real-time PCR; IFS, isoflavone synthase gene

P057

QPCR-based miRNA Profiling with High Specificity
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A miRNA QPCR-based quantitation method was developed using polyadenylation to add a polyA tail to miRNA followed by reverse transcription (RT) and QPCR (Shanfa, L, et al. 2005. Plant Cell. 17(8): 2186–2203). We have expanded on this method to develop an assay that discriminates between miRNAs that differ by as few as a single nucleotide as demonstrated by less than 1% cross reactivity between all human let-7 family members. The method was validated using 2-250ng input total RNA and resulted in a linear signal when quantitating miRNA of varying abundances. A single polyadenylation and RT reaction allows for profiling of 300-6,000 different miRNA resulting in extensive miRNA profiling from a very small amount of total RNA. The method was used to quantitate miRNA in total RNA from a variety of sources including FFPE tissues and in cell lysates. Further validation of this method identified differentially expressed miRNA in a matched set of normal colon and adenocarcinoma RNA from the same donor. In this study, 50 different human miRNA were profiled based upon their potential roles in cancer and development.
This QPCR-based method allows for highly specific and sensitive miRNA expression profiling from small amounts of RNA to identify miRNA of diagnostic, prognostic, and therapeutic value in a variety of diseases.

P058

Stability of microRNA in partly degraded RNA from lung samples
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MicroRNA has gained a lot of attention recently, due to its involvement in epigenetic regulation of gene expression. Expression profiles of these small non-coding RNAs have also been used for different diagnostic purposes. The stability of microRNA has previously been tested in formalin-fixed paraffin-embedded tissue and has shown to be relatively high (Li et al., 2007). The small size of microRNA could be a possible explanation for a greater tolerance to degradation (Fleige et al., 2006). We tested the stability of microRNA in partly degraded RNA isolated from lung tissue as little is known about obtaining reasonable qRT-PCR data of microRNA expression from partly degraded RNA. Lung tissue from three healthy pigs were cut into six pieces of 1cm x 1cm and stored at room temperature in Petri dishes in 0 h, 1 h, 8 h, 24 h, 48 h, and 72 h. To the given time RNA Later (Invitrogen) were added to stabilise the RNA. Total RNA including small RNA was extracted using TRI Regent (Sigma) and the integrity of the RNA was determined using two different chips (RNA Nano and Small RNA) on the Agilent Bioanalyser. The relative concentration of three well described reference genes (B2M, β-actin and GAPDH) was compared with the relative concentration of three putative microRNA reference genes: mir-23a, mir-26a and mir-34a. A correlation coefficient of 0.87 was found between RNA degradation (RNA integrity number (RIN)) and time (h), confirming RNA degradation within the lung tissue at room temperature. During the time span of this study RIN decreased from 9.0 (±0.12) at time 0 h to 4.7 (±1.57) at time 72 h. Electropherograms of small RNA confirmed these results, ranging from high quality RNA samples, with a clear tRNA peak at time 0 h to highly degraded samples after 72 h. The concentration of mir-23a, mir-26a and mir-34a was found to be more or less stable within the first 24 h, whereas concentration of the three mRNA reference genes was found to decrease within less than 8 h. Previous studies have found RNA stability to differ between tissues due to variation in type and quantity of active ribonucleases as well as differences in tissue structure (Schoor et al., 2003; Seear and Sweeney, 2007). In this study we tested the stability of microRNA and mRNA in partly degraded RNA isolated from lung tissue. Initial results indicate that mir-23a, mir-26a and mir-34a are relatively stable at room temperature in lung tissue within the first 24 h.

P059

MicroRNAs as potential biomarkers in the differential diagnosis of Burkitt's lymphoma vs diffuse large B-cell lymphoma

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Purpose - A clear-cut diagnosis of Burkitt's lymphoma (BL) and its differentiation from diffuse large B-cell lymphoma (DLBCL) is of great clinical importance, as therapies for BL and DLBCL differ and BL treatment should be promptly initiated. MicroRNAs - short, non-coding RNA molecules have been implicated in different pathological processes, including cancer development and progression, and an aberrant expression of numerous microRNAs has been found in multiple human tumor types.
The aim of presented study was to investigate the potential of selected miRNAs, including miRNA-155, as biomarkers for the differential diagnosis of BL vs lymphoma DLBCL.
Patients, Materials and Methods – Clinical samples from adult BLs and DLBCLs were characterized regarding morphologic features, immunophenotype (flow cytometry) and cytogenetic profiles according to the recent lymphoid malignancies WHO classification (2008). All miRNA measurements were performed using TaqMan MicroRNA assays (AppliedBiosystems) and small nuclear RNA, RNU6 was assayed for normalization.
Results - We showed that the expression of miR-155 is absent or significantly lower in adult BLs, contrary to DLBCLs. In addition, our initial experiments showed a good correlation between the expressions of miR-155 and, it's precursor, BIC in BL pathological samples. The relationships of the observed microRNA-155 expression pattern with the clinical data as well as with the cytogenetic and immunophenotype profiles of the analyzed cases will be presented.
Conclusion - Our findings highlight the importance of microRNA-155 as a potential biomarker in the differential diagnosis of Burkitt's lymphoma vs diffuse large B-cell lymphoma, and provide new insights into the role of miR-155 in the pathogenesis of the aggressive B cell lymphomas.

P060

MicroRNA expression profiling in the pig developing brain

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MicroRNAs are small, non-coding RNAs that are known to regulate the gene expression at post transcriptional level. MicroRNAs are believed to play an important role in the control of various developmental and physiological processes. Central nervous system (CNS), developing brain in particular, is proved to host an impressive diversity of microRNAs. Most of the microRNA expression profiling is done in humans or mice however there is a need for information within microRNA biology in other mammals. Domestic pigs are considered as good animal models for human related neurological studies because pigs brain development and brain growth curve is very similar to humans. Moreover, relatively small number of microRNAs is annotated in domestic pig, and most of them are in silico predicted.
In the present study, microRNA expression profiling with use of microRNA microarray and qPCR was performed on porcine developing brain. Our results show that microRNA expression is regulated in tissue as well as in developmental stage specific manner. We have found numerous tissue and developmental stage specific microRNAs in the pig brain. The different levels of expression of particular microRNAs in foetal versus postnatal samples, confirms their important role in the regulation of developmental and physiological processes that take place along the brain development. Furthermore this study supports the belief that microRNAs act as posttranscriptional switches that regulate gene expression when required.

P061

Highly sensitive and specific LNA™-enhanced real-time PCR system for microRNA expression analysis

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MicroRNAs (miRNAs) comprise a family of highly conserved small non-coding RNAs (~22 nt). As regulators of post-transcriptional gene expression, miRNAs play an essential role in many parts of development, differentiation, and physiological processes. The accurate and specific expression analysis of miRNAs is complicated by their short length and sequence similarities between miRNAs in the same family. The specific quantification of a 22 nt sequences with single nucleotide mismatches is a significant challenge. We have developed a highly sensitive real-time PCR method for quantification of miRNAs. One of the advantages of the Locked Nucleic Acid (LNA™) technology is that very short high-affinity miR-specific primers can be designed, thus working under general PCR conditions. The LNA™ primer design enables a simple and robust two-step method employing two different miRNA-specific primers: a miRNA-specific RT primer is employed in the first-strand cDNA synthesis, and for the following SYBR® Green-based quantitative PCR detection, a LNA™-enhanced primer targets the miRNA sequence at the opposite end. Hence, the method offers accurate quantification of specific miRNAs directly from total RNA. The miRCURY LNA™ microRNA PCR system offers a high dynamic range with a linear readout of miRNA concentrations spanning more than 8 orders of magnitude, enabling detection of as few as 10 RNA copies. The LNA™ enhanced primers allow discrimination between closely related miRNAs and between mature and precursor miRNAs.

P062

microRNA expression analysis by LNA™-enhanced real-time PCR

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MicroRNAs (miRNAs) comprise a family of highly conserved small non-coding RNAs (~22 nt). As regulators of post-transcriptional gene expression, miRNAs play an essential role in many parts of development, differentiation, and physiological processes. It is now established that altered miRNA expression profiles are associated with a number of different diseases including heart disease, neurological disorders and human cancers. This suggests the use of miRNAs as a novel class of biological important biomarkers for disease diagnosis and prognosis.
The study of expression and functional effects of miRNAs is complicated by their small size and limited availability of sample. We have developed a highly sensitive real-time PCR method for quantification of miRNAs. The LNA™ primer design enables a simple and robust two-step method employing two different miRNA-specific primers: a miR-specific RT primer is employed in the first-strand cDNA synthesis, and for the following SYBR® Green-based quantitative PCR detection, a LNA™-enhanced primer targets the miR sequence at the opposite end. The miRCURY LNA™ microRNA PCR system offers the possibility for highly sensitive and specific quantification of miRNA expression levels from total RNA.
In addition, a panel of endogenous control primer sets has been developed for accurate and sensitive quantification of small nucleolar RNAs, U6 snRNA and 5S rRNA. These can be used as normalizers, allowing comparison of miRNA expression levels over a range of different samples. However, the endogenous controls must always be empirically validated for each study. Even very small changes in miRNA expression levels, e.g. in comparing different disease stages, might be biologically significant. Reliable normalization is therefore critical when analyzing differences in miRNA expression. We show an example of how the use of a poor normalizer can lead to incorrect conclusion when studying differential miRNA expression.
Finally, we demonstrate the excellent correlation between miRNA expression analysis using miRCURY LNA™ Arrays and the miRCURY LNA™ microRNA PCR system.

P063

Influence of total RNA integrity on microRNA quantitation

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The analysis of RNA quality is a valuable tool in classical gene expression profiling via qRT-PCR and microarray analysis. This technique may also be integrated in the routine analysis of new applications like the investigation of miRNA expression. Agilent Technologies offers a new application for the 2100 Bioanalyzer making it possible to analyze small RNA with the lab-on-a-chip technology. By now this chip provides the only possibility to quantify miRNA in absolute amounts [pg] and as a percentage of small RNA [%]. Ongoing RNA degradation is accompanied by the formation of small RNA fragments and though possibly influences the miRNA quantitation leading to an overestimated miRNA amount. Aim of the study was to investigate the influence of artificially caused RNA degradation on miRNA quantitation and miRNA expression.
RNA and miRNA were extracted from different bovine tissues [liver, muscle, white blood cells (WBC)] in six replicates (n=6). Total RNA quantification was done using the NanoDrop. RNA quality analysis and miRNA quantitation were done with the 2100 Bioanalyzer. The measurement of the gene expression pattern for mRNA and miRNA was undertaken with real-time qRT-PCR.
Results for miRNA quantitation showed a highly significant increase in apparent miRNA percentage following ongoing RNA degradation for all investigated samples. A significant rise in apparent miRNA concentration was demonstrated for liver and WBC samples. Gene expression analysis showed a highly significant negative correlation for the CT values and the RIN.
In conclusion, the determination of miRNA quantity with the Bioanalyzer is just reliable for samples with good RNA quality. With increasing total RNA degradation level an overestimation of the miRNA amount occurs. The performance of miRNA qRT-PCR is dependent on the template quality as well as the performance of mRNA qRT-PCR.

Diagnostic & Molecular Markers (agri-vet)
Location: poster room (mensa)

P064

Detection and quantification of bovine, ovine and caprine milk percentages in dairy and soybean products using isoelectric focusing of gamma-caseins and Real-time PCR

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Dairy products made from ewes' and goats' milk are of considerable economic importance. However, the substitution of these milks for cows' milk is a fraudulent practice in the dairy industry. The seasonal changes and the much lower milk yield of ewes and goats, together with the much lower price of bovine milk are the main reasons for this adulteration. Consequenctly, an adequate methodology is required to control authenticity of dairy products. Moreover, soybean dairy-like products, which are an alternative for people suffering from an allergy against milk proteins, have to be checked to prevent the potential adulterations resulting from the addition of casein and/or whey proteins to these products and their adverse effects on allergic people.
The objectives of this study were the qualitative detection as well as the quantitative determination of cow's milk percentage in dairy and soybean products. Standard mixtures of milk from different species as well as model cheeses of different ages were used as references. Species identification was performed using different electrophoretic methods, and by conventional polymerase chain reaction (PCR) as well as quantitative real-time PCR using species-specific primers. Applied methods were evaluated regarding their applicability for the detection and quantification of cows' milk in mixed cheeses. In addition, soybean milk was spiked with different amounts of bovine milk to enable quantitative analysis of cow's milk percentages in soybean products.
Urea-polyacrylamide gel electrophoresis of caseins was restricted to the adulteration control of milk only. The official EU reference method (No 213/2001), which is based on the IEF of gamma-casein fractions, was a reliable tool to detect cows' milk even in matured cheeses made from milk of other species. Usually, the this method is performed as a qualitative technique using reference samples, which are certified with 0% and 1% of cow's milk, respectively. However, after densitometric evaluation of gamma-caseins, a quantitative estimation of cow's milk percentage was obtained in mixed cheeses. Conventional PCR was shown to be a qualitative method, although a certain semi-quantitative estimation could be achieved in some cases. Real-time PCR proved to be a high-sophisticated technique, which enables the quantitative determination of the cow's milk percentage in mixed cheeses manufactured from milk of different species, but turned out to have an unexpected high error probability. This was probably due to the fact that DNA-based methods are to be applied for quantitative adulteration control of mixed cheeses with extreme care, only!
Thus, analytical procedures used were appropriate for the qualitative detection of cows' milk in dairy and soybean products. However, quantitative results in adulteration control have to be understood as approximate values, and authentication of mixed cheeses still remains a challenge for food analysts.

P065

Development and transferability of medic EST derived SSR markers across four food grain legume species.

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Expressed sequence tags (ESTs) derived SSRs or EST-SSRs are valuable markers which can be generated and used from closely related genera. For plants such as grain legumes species lacking extensive genomic DNA sequence data, using these markers would be an economical method for the development of markers for diversity analysis. This study reports the development of SSRs from large EST databases of Medicago truncatula and evaluates the possible transferability across 10 genotypes of each of four food grain legume species namely Cicer arietinum; Lens culinaris; Vicia faba and Pisum sativum. Ests in the medicago database were searched from NCBI´s dbEST and 30 PCR primers were designed based on di-, tri and tetra simple sequence repeats (SSR) motifs and tested on 10 genotypes of each species together with four genotypes of medics. Polymorphisms were evident both at the genotype level and also between legume species. Thus, the study provides insights about those EST-derived SSRs in grain legume species that are useful for marker development due to their polymorphism and transferability.

P066

qPCR monitoring of cholesterol synthesis regulation

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Introduction: Milk cholesterol level regulation is a complex mechanism involving the intervention of many metabolic pathways, including absorption in the intestine, liver and mammary gland biosynthesis and transport mechanisms through blood and cellular membranes. In vegetarian organisms it can be hypothesized that, as diet contains scarce amounts of this component, the de-novo synthesis pathways play the crucial regulation step in the whole homeostasis.This study aims at the characterization of the liver cholesterol synthesis and its regulation during the lactation cycle of *Bos taurus* by using qPCR.
Materials and methods:Liver biopsies were obtained from 16 adult Brown Swiss cows at different time points before and after parturition (weeks -2, 0, 2, 4, 8) and RNA extracted. After reverse transcription, the expression of three rate limiting genes from the cholesterol synthesis route (HMGCoA-reductase, HMGCoA-synthase and FDFT) and three regulation factors (SREBP1,

SREBP2 and SCAP) was measured by qPCR. All experiments were performed using the LightCycler SYBRGreen technologie (Roche Diagnostic). Expression data was normalized using three housekeeping genes in form of a normalization index (Ubiquitin, GAPDH and ß-actin).
Results: A coordinated regulation of gene expression at the onset of lactation was demonstrated, most probably as an image of the immense changes in metabolism of this crucial molecule that the bovine organism has to face immediately after parturition. A significant up-regulation of the SREBP resgulation pathway occured at the first stages after parturition (weeks 0 and 2) and was followed by the correspondent increase in synthesis enzymes HMGCoA Reductase and FDFT expression during the following time points (weeks 2 to 8). HMGCoA synthase showed similar tendencies, although non-significant and non-correlated with the other studied factors, so it can be hypothesized that this crucial enzyme is subjected to different regulation mechanisms.

P067

Development of a real-time PCR for detection of Mycoplasma felis in cats

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Infection with *Mycoplasma felis* is associated with conjunctivitis, respiratory tract disease and arthritis in domestic cats. To enable intervention with antimicrobials targeting mycoplasma, accurate and rapid diagnosis of these infections is necessary. To this end we have developed a real-time PCR protocol for detection of *Mycoplasma felis* based on dual-labeled fluorogenic probe technology targeting the gene for elongation factor Tu (*tuf*). Sequencing this gene in the type strain (CO) and clinical samples of *M. felis* as well as type strains of other mycoplasma species allowed us to target regions that were species-specific while highly conserved within *M. felis* . Using the same panel of mycoplasma type strains and clinical strains of feline origin to evaluate the developed assay, we found a detection limit in the order of 10fg of *M. felis* genomic DNA and no cross-reaction with other mycoplasmas. We also compared the assay to an available conventional PCR on a panel of conjunctival swabs from cats with clinical signs of ocular infection (n=100). We found the new assay to be more sensitive and specific in this setting, detecting one additional positive while rejecting a sample found positive by conventional PCR which carried a *tuf* gene identical to that of the type strain of *Mycoplasma cynos* (H831). We suggest that the developed assay might be a useful tool for research and routine diagnostics.

P068

Differential temporal expression of staphylococcal enterotoxins and enterotoxin-like superantigens during in vitro growth

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Staphylococcal food poisoning (SFP) is an intoxication resulting from ingestion of foods containing one or more preformed enterotoxins from Staphylococcus aureus. Staphylococcal enterotoxins (SEs) and enterotoxin-like proteins (SEls) are a family of structurally related proteins belonging to the pyrogenic toxin superantigen family. To date, 21types have been described (SEA-SEE, SEG-SEI, SEIJ-SEIV).
To better understand how those genes are expressed during a growth cycle, we developed a qRT-PCR procedure to determine their temporal expression pattern. PCR assays that allow for the screening of 18 se/sel genes were designed and a panel of strains was examined for their distribution. A total of 28 strains displaying various combinations of se/sel genes were selected for further kinetic mRNA study.
To examine the impact of growth phase on the dynamics of se/sel transcription, strains were cultured at 37°C in BHI broth and RNA harvested at 4 time points from mid-exponential to late stationary phase. Expression of each gene was measured by qRT-PCR and relative expression ratios were calculated. Three reference genes whose expression was found to be few affected by growth conditions were selected with the GeNorm software and quantified at the same time. Normalization was provided using the geometric mean of these latter genes.
Different patterns of expression were found depending on the species and genes studied. seb and sec mRNA abundance were found to be transiently upregulated at the transition into stationary phase, a characteristic of their regulation by the accessory gene regulator (agr) system. sed was also significantly induced at the end of the exponential growth phase but in lesser extend. In contrast, most se/sel transcript levels remained relatively constant or decreased from exponential to stationary growth phases.

P069

Expression of VEGF-B isoforms and neuropilin-1 in bovine ovary during different physiological stages

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Vascular endothelial growth factor-B (VEGF-B) is a member of the VEGF family, a system of growth factors that is known to regulate blood vessel angiogenesis. Neuropilins (nrp.-1 and nrp.-2) are co-receptors for VEGF-R and can thus enhance VEGF effects. VEGF-B can only bind to nrp.-1. The aim of this study was to characterise the expression patterns of the mainly anti-apoptotic factor VEGF-B and its splicing isoforms (VEGF-B167, VEGF-B186) in bovine ovary during final follicle maturation, corpus luteum (CL) function and regression (induced luteolysis) as well as the expression of nrp.-1. Experiment 1: Antral follicle classification occurred by follicle size and oestradiol-17β (E2) concentration in follicular fluid (FF) into 5 groups (<0.5, 0.5-5, 5-40, 40-180 and >180 ng/ml). Granulosa cells (GC) and theca interna (TI) were investigated separately. Experiment 2: CL were assigned to the following stages; days 1-2, 3-4, 5-7, 8-12, 13-16, >18 (after regression) of estrous cycle and of pregnancy (month 1-2, 3-5, 6-7, >8). Experiment 3: Induced luteolysis, cows on days 8-12 were injected with PGF2α analogue and CL were collected by transvaginal ovariectomy before and 0.5, 2, 4, 12, 24, 48 und 64h after PGF2α injection. The expression of mRNA was measured by qRT-PCR and the concentrations of the hormones in FF by ELISA. In GC and TI of antral follicles only VEGF-B186 could be measured. Its mRNA levels in GC increased significantly in larger follicles. The same expression patterns could be observed for nrp.-1. In the TI VEGF-B186 was constantly up regulated during follicle maturation, but declined in follicles with E2 >180 ng/ml. In CL during estrous cycle transcripts of both VEGF-B isoforms declined from days 3-4 to 13-16, but were up regulated significantly in CL >18 days. During pregnancy mRNA of both isoforms were constantly expressed. During induced luteolysis VEGF-B186 increased significantly 24h after PGF2α injection, while nrp.-1 decreased at this point of time. Both factors showed constantly high expression during functional luteolysis. In conclusion, the temporal mRNA expression of VEGF-B isoforms and nrp.-1 in antral follicles and CL of induced luteolysis, as well as VEGF-B isoforms in CL of estrous cycle and pregnancy, suggests them to be important mediators of follicle maturation as well as CL formation, function and regression in bovine ovary.

P070

Direct Quantification of thermophilic Campylobacter species in chicken rinse samples using real-time PCR

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Quantitative microbial risk assessment is becoming more and more important in diagnostics of food-borne pathogens. It can be based on the existence of virulence factors or simply on the number of pathogens per food. It has been shown that conventional microbiological methods are not suitable for quantification because of low sensitivity, influences of background flora and labor-intensity. Especially if bacteria are difficult to culture counting of colony-forming units is not reliable. Using real-time PCR for quantification, however, the number of cells can be determined more reliably as each cell, dead or alive, will be detected. One of the most common causes of food associated bacterial gastroenteritis worldwide are thermophilic Campylobacter species. Therefore, quantitative risk assessment of these bacterial species is of great importance. In this study, 44 chicken rinse samples were analyzed. Presence and number of cells of Campylobacter spp. were determined without pre-enrichment of

the samples applying plate counting in comparison to real-time PCR. A published real-time PCR assay that specifically detects C. jejuni, C. coli and C. lari was applied. For quantification of cell numbers inactivated and stabilized Campylobacter cells were used as reference material for the quantitative PCR. This reference material is used for the calibration of the data with respect to the total efficiency for the entire process from DNA preparation to the quantitative real time PCR. It was shown that the real-time PCR was useful for quantitative detection of thermophilic Campylobacter spp. in chicken rinse samples. Compared to the cultural method, the results are provided faster and can be performed independently of the background flora present in the sample. The application of a simple to use precalibrated standard with known cell number accurately determines the number of pathogens in a food sample.

P071

Effect of apple pectin on gut microbiota - qPCR in applied microbiology

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This study was part of the large European project ISAFRUIT aiming to reveal the biological explanations for the epidemiologically well-established health effects of fruits. The objective was to identify effects of apple and apple product consumption on the composition of the cecal microbial community in rats, as well as on a number of cecal parameters, which could be influenced by a changed microbiota.
Principal Component Analysis (PCA) of cecal microbiota profiles obtained by PCR-DGGE targeting bacterial 16S rRNA genes showed an effect of whole apples in a long-term feeding study (14 weeks), while no effects of apple juice, purée or pomace on microbial composition in cecum were observed. Administration of pectin derived from apples resulted in considerable changes of these DGGE profiles.
A 2-fold increase in the activity of beta-glucuronidase was observed in animals fed with pectin (7% in the diet) for four weeks, as compared to control animals (P<0.01). Additionally, the level of butyrate measured in pectin-fed animal was more than double of the corresponding level in control animals (P<0.01). Sequencing revealed that DGGE bands, which were suppressed in pectin-fed rats, represented Gram-negative anaerobic rods belonging to the phylum Bacteroidetes, whereas bands that became more prominent represented Gram-positive anaerobic rods belonging to the phylum Firmicutes, and specific species belonging to the Clostridium Cluster XIVa. These findings indicate that consumption of apple pectin increases the population of butyrate- and beta-glucuronidase producing Clostridiaceae in the rat gut.
The quantitative changes in bacterial composition were verified by quantitative real-time PCR using SybrGreen. All results were calculated as ratios of relative expression levels to HDA expression levels in order to correct data for differences in total DNA concentration between individual samples.The HDA primer set amplifies the V2-V3 region of the 16S ribosomal DNA gene, a well conserved bacterial marker region.
The RT-PCR data will be presented at the symposium as an example of the application of quantitative PCR in the data analysis of prokaryotes.

P072

Expression of estrogen receptor alpha and beta in reproductive tissues of male growing piglets

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Objectives: Exposure to estrogens is critical during juvenile development in males when low endogenous hormone levels prevail. The sensitivity towards sex steroids might be of specific relevance for disturbances associated with subsequent reproductive failure. We characterized the expression of estrogen receptors alpha (ESR1) and beta (ESR2) during different points of time in male pre-pubertal piglets.
Methods: Male siblings of German Landrace sows (n=6) were slaughtered at <1h, 11 and 48 days after birth. Estradiol-17β (E2) concentration in plasma at slaughtering was measured using a competitive ELISA. Testis, corpus epididymis and prostate tissue were sampled. RNA was extracted for quantitative real-time PCR of ESR1 and ESR2. ESR1 protein was determined by immunohistochemistry.
Results: E2 concentration was highest in newborns (78.3±14.8 pg/ml), intermediate at the age of 11 days (9.2±2.2 pg/ml) and lowest in pre-pubertal piglets (1.8±0.5 pg/ml). ESR1 transcripts were detected in all tissues analyzed. ESR1 transcript abundance in corpus epididymis was 2.1-fold and 1.7-fold on days 11 and 48, compared to newborns (P=0.01), while prostate and testis showed no significant differences over time. ESR1-protein increased in prostate from <1h to 48 days after birth in smooth muscle cells and glandular epithelial cells. In testis, ESR1 was mainly localized to premature seminiferous tubules, in epididymis to epithelial cells. A methylation analysis of the ESR1 promoter using Pyrosequencing-technology is undertaken attempting to explain differential gene expression. ESR2 transcripts were present in testis at the three distinct time points, but below the detection limit in prostate and epididymis at 11 and 48 days after birth, respectively.
Conclusion: In summary, growing piglets exhibit very distinct endogenous levels of E2 over the first few weeks of lifespan offering an interesting model to study effects of endogenous estrogens on development. Male reproductive tissues are distinctly regulated during pre-pubertal growth with respect to ESR1, which may be associated with tissue-specific differences of sensitivity to estrogens.

P073

GENE EXPRESSION OF PPARGC1A AND SEVERAL DOWNSTREAM TARGET GENES IN PORCINE BACKFAT AND LONGISSIMUS DORSI MUSCLE

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Peroxisome proliferator-activated receptor γ coactivator 1α (PPARGC1A) is a versatile coactivator and is of vital importance to energy and fat metabolism. Based on these functions, it not only is an excellent candidate gene for meat quality in pigs, but also is of great interest for human research. The information on the in vivo gene expression and relationship between PPARGC1A and its downstream target genes is however very limited, especially in the pig. In this study, the gene expression pattern of PPARGC1A and 10 of its putative target genes was determined in backfat and longissimus dorsi muscle (MLD) samples from 20 pigs, by real-time PCR. Samples were preserved in RNA-later (Sigma-Aldrich), crushed to powder with liquid nitrogen and stored at -80°C until use. The Aurum Fatty and Fibrous Tissue RNA Isolation Kit (Bio-Rad) and iScript cDNA Synthesis Kit (Bio-Rad) were used for total RNA isolation and cDNA synthesis, respectively. Real-time PCR was conducted with the Platinum SYBR Green qPCR Supermix UDG (Invitrogen) on an iCycler iQ Real-Time PCR Detection System (Bio-Rad). Evaluation of reference gene expression stability by geNorm indicated that normalisation with ACTB, TBP and TOP2B provided reliable mRNA expression results.
Except for UCP3 and LPL, a very significant expression difference was found between backfat and MLD for all genes ($P < 0.01$). Statistical analysis indicated that there was a strong genetic regulation of the mRNA expression of several target genes. A positive correlation with PPARGC1A gene expression was found for CPT1B, GLUT4, PDK4 and TFAM ($P < 0.0001$). A negative correlation was found for UCP2, FABP4, LEP ($P < 0.0001$), and TNFα ($P = 0.0071$). No significant correlation was detected for UCP3 and LPL. In conclusion, these data suggest a clear impact of PPARGC1A on energy and lipid metabolism in vivo in the pig through several of these downstream target genes.

P074

High Resolution Melt analysis of _Brachyspira_ for identification of *B. hyodysenteriae* and *B. pilosicoli*

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We will show a new High Resolution Melt (HRM) assay for the identification of the genus *Brachyspira* by preamplification with

primers specific for *B. hyodysenteriae, B. pilosicoli, B. murdochii, B. Innocens, B. intermedia* and *"B. suanatina"* and subsequent HRM analysis to differentiate between *B. hyodysenteriae, B. pilosicoli* and all others. The methodology is simple and straight I PBS andμforward, one loop full of bacteria is suspended in 200 boiled for ten minutes, the lysate is centrifuged at 10 000 x g for 10 minutes and the supernatant is transferred to a clean tube. To investigate the potential of the assay in routine diagnosis of pig diarrhoea, stool samples were spiked with different concentrations of *Brachyspira* cells. Total DNA from the stool samples were extracted with QIAamp DNA Stool Mini Kit with the QIAcube (QIAgen). DNA from reference strains were extracted with Easy-DNA (Invitrogen). All DNA samples were analysed with the same pair of primers giving an 88 bp amplicon from 23S rDNA in RotorGene 6000 (Corbett). Different dilutions of reference strains were used as positive controls of the different species in each run.
The melt and HRM analyses of samples were visually compared to the melting plots and HRM analyses of reference strains. *Brachyspira* DNA will result in three melting peeks which can be identified as belonging to *B. hyodysenteriae, B. pilosicoli* or other *Brachyspira. sp.* The normalised plot of the HRM analyses shows three separate curves. Reaction efficiencies and detection ranges for individual *Brachyspira* species as well as the validation regarding negative controls are shown. The Danish Veterinary Institute is presently engaged in developing new methods for the diagnosis of the causative agents in pig diarrhoea. The HRM assay has the promise of becoming a valuable diagnostic tool in the laboratory.

P075

LUX PCR assay and comparison with SYBR-GREEN PCR and TaqMan PCR formats for detection of DNA virus (PCV2)

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At present several formats of real-time PCR were developed with application for diagnostics assays of infectious diseases in human and veterinary medicine. The LUX PCR (light upon extension PCR) is relatively new technique of real-time PCR which is based on the use of two primers where only one primer is labeled near 3'-end with a fluorophore molecule. We have developed for the first time LUX PCR assay for the detection of porcine circovirus type 2 (PCV2) infecting pigs. LUX PCR assay amplifying a 119 bp fragment of PCV2 ORF1 detected at least 20 copies of viral DNA inserted into recombinant plasmid. The dynamic range of this quantitative assay covered range from 200 to 100 000 000 copies of viral genome. The assay specifically detected only PCV2 with negative results for other swine viruses (CSFV, PRRSV, PCV1).
The SYBR-GREEN PCR with primers CF8/CR8 (1) amplifying a 263 bp fragment and TaqMan PCR system (2) modified in our laboratory amplifying a 64 bp fragment were used for the comparison with LUX PCR assay. The assays were tested on clinical samples from PCV2 infected pigs. DNA was isolated from samples by the Chelex method. The amplification curves were characteristic for different types of real-time PCR techniques and Ct values for the same sample were also different from assay to assay. The lowest numerical Ct values were obtained in SYBR-GREEN PCR, than in TaqMan PCR, the highest Ct values were observed for LUX PCR. This observation corresponds to basic principle of the detection of fluorogenic signal during amplification process. However the threshold values in all three assays provided linear calibration curve with similar quantification of viral copies for a particular sample (tested in range 100 to 10 000 copies/25 ul of assay volume). Reproducibility of results tested in inter-experiment evaluation was relatively stable varying not more than a half order of viral copies. Negative samples provided values not more than 0 to 40 copies (in SYBR-GREEN and TaqMan PCR). All these data suggest that the evaluation of results for a particular real-time PCR assay should be carefully interpreted. LUX PCR and TaqMan PCR systems are in principle, and in our experiments too, more specific than SYBR-Green PCR. LUX PCR as methodologically simple technique is a method of choice for the laboratory detection and quantification of PCV2 in clinical samples.

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P076

Optimisation of a qRT-PCR assay to determine Vp1 expression in relation to pre-harvest sprouting tolerance in triticale

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Pre-harvest sprouting (PHS) is a major problem in triticale production during rainy harvest periods, inducing a severe loss in grain yield and quality. Due to the influence of genotype, the environmental conditions and their interaction, the only solution to this problem is to breed PHS tolerant cultivars. Several field-independent methods exist but seem to be unreliable to select for PHS tolerant triticale varieties. The aim of this study is to obtain more insight in the complexity of PHS through a molecular genetic approach.
Typically PHS sensitive varieties lack adequate dormancy levels to prevent early sprouting during wet harvest periods. In literature many genes are cited for their important role in embryo development, dormancy induction and dormancy maintenance. These genes are mostly involved in pathways of plant hormone synthesis and catabolism or alpha-amylase synthesis and degradation. Most of these genes are genetically analysed in wheat, rice, barley, maize, rye or *Arabidopsis thaliana* and may be suitable for use in a qRT-PCR assay. As dormancy is installed during kernel development, an integrated study during kernel development may reveal some factors which can be useful to select for PHS tolerant varieties.
Plants of a PHS sensitive and a PHS tolerant variety of both wheat and triticale were grown in a growth chamber under controlled conditions from flowering to ripeness. At regular days post anthesis kernels were harvested for germination tests, plant hormone detection and RNA extraction. After RNA extraction and DNase treatment first strand cDNA was prepared and could be used for qRT-PCR with SYBR Green chemistry. For a relative quantification of the expression level of the intended target genes, a proper selection of reference genes is necessary for an adequate normalisation. In this study eleven reference genes were selected and checked for a stable expression pattern during kernel development in both wheat and triticale. The four reference genes with most stable expression pattern were selected using the GeNorm application. The first results indicate that the genes coding for beta-tubulin, ubiquitin conjugating enzyme, elongation factor and cytoplasmic malate dehydrogenase perform the most stable expression level and can be used as references.
As a first gene of interest many attention was paid to the 'viviparous' *Vp1* -orthologue. In wild oat and wheat a correlation between the expression level of the *Vp1* gene and the level of seed dormancy is described. However, until now our study could not confirm this correlation in mature seeds with use of the qRT-PCR technique, probably due to a low expression level of *Vp1* in mature embryos. The above described alternative approach may contribute to a better understanding of the expression profile of *Vp1* and other target genes during kernel development of triticale specifically, and PHS in general.

P077

Expression of some angiogenic factors in bovine follicles before and after LH surge

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Angiogenesis is defined as the generation of new blood vessels through sprouting from already existing blood vessels in a process involving different angiogenic factor families. The aim of this study was to evaluate the expression patterns of hypoxia-inducible factor-1 alpha (HIF), VEGF and angiopoietin (ANPT) system members (VEGF isoform 120, 164, 188, VEGFR-1, VEGFR-2, ANPT-1, ANPT-2, and Tie-1, Tie-2 receptors) in time defined follicle classes before and after GnRH application and after ovulation in cow. Ovaries containing preovulatory follicles or new corpora lutea (CL) were collected at 0h, 4h, 10h, 20h, 25h

(follicles) and 60h (CL day 1-2) relative to injection of GnRH (n=5/group). For better characterization of follicle classes, estradiol-17beta and progesterone were determined by EIA in follicular fluid (FF). The mRNA of HIF decreased in follicles only during the periovulation phase, followed by a significant increase in early CL tissue. Transcripts of all VEGF isoforms were upregulated 4h after GnRH followed by lowest expression around ovulation. The VEGF peptide content in FF increased 25h after GnRH. All VEGF isoforms as well as their receptors were upregulated again after ovulation. ANPT-1 mRNA decreased significantly in follicles during LH surge. ANPT-2 decreased 10h after GnRH and in the follicle group during ovulation. Tie-1 and Tie-2 mRNA expression decreased in follicle group during ovulation, with a further increase in early CL tissue. It is likely that the decrease of ANPT-1 and therefore the increase of the ANPT-2/ANPT-1 ratio during the LH surge is a basic mechanism of vascular remodelling in follicles during periovulation. In conclusion, the temporal expression pattern of angiogenic factors (HIF, ANPT and VEGF family members) during periovulation suggests them to be important mediators of the ovulatory process and the early CL formation (angiogenesis) in the bovine ovary.

P078

Quantitative detection methods for evaluating induction of disease resistance against Verticillium dahliae correlated with increased defense responses in tobacco plants treated with Coactyl

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Introduction – Verticillium wilt remains one of the most serious soil borne diseases worldwilde. An array of different strategies to reduce the consequences of pathogen pressure is available. Among these methods, application of natural compounds, which stimulate locally and systemically plant defense reactions, is becoming an industrial alternative to the application of chemicals with deleterious side effects. Our objective was to investigate, through the quantitative and very sensitive real-time RT-PCR technology, the potential of Coactyl™, composed of humic acid associated to a phenolic acid, to act as a plant defense inducer conferring resistance to Verticillium dahliae wilt in tobacco used as a model. Preliminary experiments showed that application of Coactyl drastically reduced the severity of V. dahliae infection, which was further quantified by real time RT-PCR. PR protein genes are well recognized tobacco molecular markers of defense activation. There are more than 10 families of these proteins, which exhibit anti-fungal and anti-microbial activities. Expression of a representative set of these genes, PR1 acid, PR2 acid and basic isoforms, PR3 basic and PR5 acid and basic isoforms, was evaluated by real-time RT-PCR.
Results - Quantification by real-time PCR of _V. dahliae _growth in planta was achived using specific primers of the internal transcribed spacer ITS of *V. dahliae* (AB353346). Sequencing amplicon clones identified only the target V. dahliae sequence and no tobacco sequence demonstrating the specificity of the reaction. Experimentally, tobacco plants were treated by Coactyl 10 days before _V. dahliae _inoculation. Then, seven days after inoculation, V. dahlia was quantified in roots, stems and leaves of infected and mock-treated plants. We measured a severe reduction of _V. Dahliae _amounts as well as a delay is plant colonization. As expected from the sensitivity of the method, _V. Dahliae _could be detected before any symptom could be observed. Decrease in _V. dahlia _infection correlated with the increased expression levels of PR protein genes, locally in roots were Coactyl was applied, and systemically, in stems and leaves.
Conclusion - First, the use of the real-time (RT-PCR/ PCR) technologies allowed to asses both the level of fungus, thus quantifying the level of disease resistance, and the level of plant defense genes, thus quantifying the level of plant defense mechanisms. Second, our results highlight the potential of Coactyl as a novel compound able to provide commercially significative resistance of tobacco against _V. dahliae _trough the activation of molecular defense responses. Preliminary disease resistance tests have shown that Coactyl is also active in other dicotylodenous plants further supporting Coactyl as a novel bioactive preparation. The molecular mode of action of Coactyl remains to be determined, however. It is currently under investigation.

P079

Rapid detection and differentiation of foodborne pathogens in the official food control using real-time-PCR

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The rapid detection and differentiation of human pathogens in food is one of the main instruments of the official preventive consumer protection policy. The cultural detection of foodborne pathogens is a time-consuming and ineffective method for testing large numbers of food or environmental samples, especially in outbreak situations and microbiological methods are not able to deliver informations about different toxin gene combinations of humanpathogenic bacteria like Clostridium botulinum, Clostridium perfringens, Bacillus cereus or Vibrio spp. But these informations are essential for a well-founded risk assement, especially for bacteria species, which exist in a pathogenic and a non-pathogenic variant.
These are the reasons, why in the last few years the Bavarian Health and Food Safety Authority has established multiplex-real-time-PCR systems not only for the detection of the “classical” foodborne pathogens like Salmonella spp. or EHEC, but also for the rapid detection and differentiation of Clostridium botulinum, Clostridium perfringens, emetic and diarrhoeic Bacillus cereus and pathogenic Vibrio spp.. The methods are used for screening without enrichment directly from the sample material, for screening after enrichment or for verification and differentiation of the isolates. Every multiplex real-time-PCR is combined with an heterologous Internal Amplification Control system based of the pUC 19-plasmid. Depending on the sample material and the kind of bacteria different DNA extraction methods are applied in routine diagnostic. Data collected in the diagnostic of gram-positive bacteria (Clostridium spp. and Bacillus spp.) from different sample material using two commercial extraction systems will be presented.

P080

Stage specific transcription Studies on Latrophilin-like protein 2 of the cattle strongyle Cooperia oncophora using Quantitative Real-time PCR

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The new anthelmintic compound emodepside has a wide range of efficacy and also resistance breaking properties. The G-protein coupled receptor (GPCR) HC 110-R of the sheep nematode *H. contortus* has been identified previously as a putative target. It’s orthologue in *C. elegans* is latrophilin-like protein 1 (LAT-1). Another latrophilin-like protein, LAT-2, is discussed as an additional receptor for emodepside in *C. elegans* . We identified the orthologous GPCRs in a subset of parasitic nematodes. Recently a dose-dependent effect of emodepside on worm development in C. elegans was shown. Also reduced emodepside sensitivity, measured via a locomotion assay, in early larval stages (L1 – L3) was detected. The time required for hatching of eggs exposed to emodepside did not significantly increase or decrease. Own studies showed via egg hatch test, that hatching is not affected in several gastrointestinal nematodes. With a view to possible differences in efficacy on different developmental stages, we analysed the transcription level of LAT-2 in eggs, mixed first and second stage larvae, infectious third stage larvae, adult males and females of the cattle strongyle *C. oncophora* . We applied 18 S rRNA, 60 S acidic ribosomal protein, ß-tubulin and Glyceraldehyde-3-phosphate dehydrogenase as reference genes for the assay. There were no significant differences to detect in the transcription level of LAT-2 across the five examined developmental stages of *C. oncophora* . This shows that no direct correlation between a reduced emodepside sensitivity in eggs and early larval stages and the presence of LAT-2 can be created here. Whether this is due to the fact, that LAT-2 is actually not involved in the mode of action of emodepside, or that other components of the signalling cascade might regulate the effect of the anthemintic remains to be investigated.

P081

Suppression of eosinophiles after ovulation alters the progesterone values of the bovine corpus luteum -Evalution of FGF and VEGF gene expression via qPCR

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The funktion of eosinophils (EOs) is unknown during the development of the bovine corpus luteum (CL). We suppressed the migration of eosinophils into the ovary before and during ovulation through the injection of cortisone (Dexamethasone (D)) to evaluate the role of EOs in the CL. Material and methodes: 10 cows were subdivided in two groups (n = 5). Oestrus synchronisation with two injections of 500 µg Cloprostenol within 10 days. 18 hours (h) later 15 mg Dexamethasone or 15 ml saline were given. Ovulation was induced 24 h later with 14 µg GnRH. Second injection of 15 mg Dexamethasone or 15 ml saline 12 h after the GnRH injection. Blood samples from the jugular vein were taken every day. EO were counted till day 7 every 24 h. The ovaries were collected at day 2 and 5 of the oestrous cycle. Protein concentrations of progesterone, fibroblast-growth-factor-2 (FGF-2) and vascular endothelial growth factor (VEGF) were measured by ELISA. The gene expression of FGF-1, -2, -7, VEGF-120, -164, -188 and the receptors FGFR, FLK and FLT were evaluated by qRT-PCR using the Rotor Gene 3000. Results: EOs counted in blood decreased significantly after the first D injecition till day 7 with no decrease in the control group. The progesterone values decreased significantly in the D group from day 8 – 17 (oestrous cycle) compared to the control group. FGF-2 protein level increased significantly at day 2 in the D group compared to day 5 and to the control group. No significant regulation was found in the control group at day 2 and day 5. VEGF protein level at day 2 of the D group decreased significantly compared to day 5 and the control group. The expression of VEGF-120, -164, FLT and FGF-2 were not significantly regulated. VEGF-188 showed a significant higher expression in the D group at day 2 than at day 5, which was not seen in the control group. The receptor FLK was significantly down regulated at day 2 in the D group compared to control group. It was also significantly lower expressed at day 5. The mRNA expressions of FGF-1 and FGFR were significantly down regulated in the D group at day 5 compared to day 2. FGF-7significantly increased in the D group at day 5 compared to day 2 and the control group at day 5. Conclusion: The lower progesterone level in the D group might be caused by an undersupply of luteal cells due to a lesser capillary density in the developing CL induced by the lower protein level of VEGF and the decrease of the FLT expression. VEGF normally acts as strong angiogenic agent in the developing CL, which seems to be suppressed during D treatment. FGF-2 is known to be an inhibitory factor of apoptosis in granulosa cells. Its high protein level during D treatment might indicate a counter regulation due to a higher risk of apoptosis induced through the lesser capillary density. It could be possible that cows with a high cortisone blood level during ovulation develop an insufficient CL leading to a lower pregnancy rate.

P082

The Development of Novel Real-Time PCR Veterinary Diagnostic Assays

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The development of validated, novel, sensitive and specific diagnostic assays for the detection of microbial and viral pathogens is a key issue in the Agri-food industry and the veterinary research/animal health care sectors. Infections caused by these pathogens result in major financial losses and their timely identification would serve to limit the spread of disease. In the Molecular Virology Laboratory we are developing a suite of multiplex diagnostic assays, based on dual-labeled hydrolysis probe technology, for the detection of selected bovine pathogens and other species specific markers. This project focuses on key areas of veterinary diagnostics; bovine respiratory infections which are a critical causative factor in mortality in adult cattle and calves, accounting for between 24-30% of all cases noted in Ireland (2007) and bovine abortion which represents a major economic loss, while also posing the risk of human zoonotic infection. Recent reports have indicated the involvement of numerous organisms in this area and disease targets selected for study include Bovine Genital Campylobacteriosis, Infectious Bovine Rhinotracheitis, Leptospira hardjo, Neospora caninum, Respiratory Syncytial Virus and Bovine Para-influenza 3 virus (PI-3). Additionally EC regulations require the species specific identification of all feed products for farmed animals entering the food chain; thus, the development of a Meat and Bone Meal composition test is also fundamental to this project. Assay conception and design for the detection of several of these targets has been completed in the lab and final validation and comparison to the current gold standard of these tests is currently underway. These assays will be rapid, economical, amenable to high-throughput and will also further serve as tools to research the pathology of the infections concerned.

P083

Use of real-time PCR methods targeted on the heat shock protein genes for the quantitation of bovine mastitis pathogens Streptococcus agalactiae, Streptococcus uberis and Streptococcus bovis in milk and food samples.

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Streptococcus agalactiae, Streptococcus uberis, and Streptococcus bovis are the major pathogens which cause mastitis in dairy herds. Since conventional methods for the detection of these mastitis pathogens are laborious and time-consuming, rapid and reliable methods are needed. In this study, we developed real-time quantitative PCR methods using primers designed from the heat shock protein genes for the quantitative detection of S. agalactiae, S. uberis, and S. bovis. Using such methods, all the target strains could be specifically and quantitatively detected in milk and meat samples. As these real-time PCR methods were used for the direct detection of mastitis pathogens in milk and food samples, the detection limit was N (N= 1–9) ×102 CFU per ml or per g of the sample. The endogenous microflora in these samples would not interfere with the detection. If a 10 h pre-enrichment step was performed, the detection limit was N×100 CFU per ml or per g. Thus, such real time PCR quantitative methods could be used for the specific and quantitative determination of bovine mastitis bacteria.

P084

Validation of a real-time PCR for quantification of Lawsonia intracellularis in porcine faeces samples

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Introduction - *Lawsonia (L.) intracellularis* is the etiologic agent of porcine proliferative enteropathy, which occurs in different forms in pigs. In live pigs, PCR on faeces samples has been reported as being a reliable and suitable technique. While conventional PCR assays give only qualitative results, it would be beneficial to evaluate the amount of *L. intracellularis* in faeces, because the association between dosage and disease has been previously published. The aim of the present study was to develop and to validate a real time PCR for the detection and quantification of *L. intracellularis* in porcine faeces samples.
Material and Methods - Specific target sequences were evaluated by comparing single genes from *L. intracellularis* with published sequences in the GenBank. A further set of primers was used to produce a larger fragment, which was flanking the target sequence 200 bp forward and reverse. This PCR-product was cloned and used as calibrator for quantification by means of ΔΔCt. The validation was performed according to international guidelines (CVMP/VICH/590/98; GL1 & CVMP/VICH/591/98; GL2, Committee for Medicinal Products for Veterinary Use). The parameters specificity, detection limit, quantification limit, linearity, range, robustness and precision including repeatability and intermediate precision were assessed by testing plasmids, several bacteria and faecal samples. Furthermore the recovery and uncertainty were evaluated. Finally, the influence of storage (time and temperature) of the faecal samples on the quantity of specific nucleotides of *L. intracellularis* was examined. All reactions included an internal positive control, were performed in triplicates and repeated three times. Statistical analyses were carried out with SAS® 9.1 (SAS

Inst., USA). Results - The analytical specificity was confirmed by the absence of positive signals when DNA from other sources than *L. intracellularis* was used as template. Sequencing of PCR products obtained from randomly selected faeces samples previously evaluated as being positive ascertained 100% homology with *L. intracellularis* (NC 008011). The detection limit was 1 copy/reaction and quantification was reliable between 10^1 and 10^7 copies/µl reaction volume. The linearity calculated by logistic regression revealed a slope of -3.329 reflecting an efficiency of 99.7% for this PCR assay. Results from faeces stored at different temperatures for a variable time showed a consistent level of copies in each sample. Inhibition was absent in the entire study. Discussion - This real time PCR described herein revealed consistent results for the certain amount of *L. intracellularis* in porcine faeces samples. In contrast to common PCR in combination with gel electrophoresis this method enhances a reliable quantification and is even more sensitive. Moreover, it was shown that storage of samples and repetition of tests by same or other investigators does not influence the outcome.

P085

Differential expression of apoptotic factors in bovine endometrium and embryos during the preimplantation period

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Bovine trophoblasts release IFN-tau, a type I IFN, as pregnancy recognition signal. Since type I IFNs exert growth inhibitory and proapoptotic actions, the expression of type I IFN receptor subunits IFNAR1 and IFNAR2 was analysed in the bovine endometrium and the respective trophoblasts during the preimplantation period. Additionally, key factors of the extrinsic and intrinsic apoptosis pathways were determined. Uteri of estrous synchronized Simmental heifers were flushed post mortem at day 12, 15, and 18 of cycle or pregnancy for the recovery of embryos and the sampling of ipsilateral endometrial tissue for quantitative RT-PCR, luminescence caspase activity assays and histochemistry. The mRNA levels of both type I IFN receptor subunits showed neither significant variations in cyclic nor pregnant animals whereas expression descended to 10.8% ± 1.20% ($p=0.04$) and 0.16% ± 0.05% ($p<0.001$) for IFNAR1 and IFNAR2, respectively in day 15 trophoblasts. Gene expression analysis of proapoptotic genes revealed a significant increase for XAF1 (4.2- and 22.4-fold at days 15 and 18, $p<0.001$, respectively) and TRAIL (11.8-fold at day 18, $p=0.006$) in pregnant animals. However, in day 18 trophoblasts XAF1 transcript levels only accounted for 0.003 % ± 0.001% when compared to gravid endometrium at day 18. For the XAF1 antagonist XIAP and for other antiapoptotic genes (Survivin, BclxL, FLIP) tested no significant changes were detected in the endometrium. Remarkably, next to abundant Survivin transcripts in day 15 trophoblasts (157-fold higher than day 15 endometrium, $p=0.002$) we detected an 82-fold increase of FasL tanscripts comparing day 15 to day 18 trophoblasts. If secreted, FasL could therefore act as additional proapototic ligand on the endometrium. However, a colorimetric TUNEL assay showed no increase in apoptotic cells numbers in the endometrium. Furthermore, expression analysis of effector caspases 3 and 7 revealed no modification in the endometrium throughout cycle and early gravidity which was consistent with luminometrically caspase activity assays in tissue homogenates. Although bovine concepti showed reduced IFN receptor expression there might be IFN-independent mechanisms for the initiation of apoptotic pathways in bovine embryos at the transcript level.

P086

Effects of Masson Pine pollen extracts on the gene expression profile of porcine ileal cell cultures

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Masson Pine pollen has been used in the traditional Chinese medicine for several hundred years and it is said to have health promoting effects. Its main application has been the treatment of disorders of the digestive system but pine pollen has also been used for a variety of other medicinal or cosmetic purposes. But only in recent years some evidence has been found that pine pollen and its compounds do have effects e.g. on inflammatory activities in mice or on TNFα expression in piglets and at least a part of these effects have been attributed to the content of polyphenols in pollen.
In the present study different extracts of *Pinus massoniana* pollen were analyzed for their effects on cell proliferation and mRNA expression levels of selected genes. Cell proliferation was analyzed using an electronic cell impedance sensing technique and relative gene expression profiles were investigated using qRT-PCR. It was found that water and 50% ethanol extracts of Masson Pine pollen in a concentration equivalent to 1% unprocessed pollen decreased cell proliferation significantly ($p<0.5$) while 100% ethanol, 80% methanol and hexane extracts had no effects on cell proliferation. At the same concentration only the 50% ethanol extract led to a significant up-regulation of the relative expression levels of the pro-inflammatory genes IL6 and IL 8 and to a down-regulation of proliferation promoter Cyclin A ($p<0.05$).
LC-ESI-MS was performed in order to characterize the compounds responsible for these effects and a number of distinct mass signals has been identified that can be found in the 50% ethanol extract but not in the aqueous extract. A detailed identification of these substances was not yet possible due to missing reference substances – with the exception of floretin and naringenin.

P087

Evaluating quantitative real time PCR (Q-PCR) data of a weakly regulated gene with kinetic analysis and the ΔΔCt method.

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An RNAi experiment knocking down a hormone receptor in the salmon louse (*Lepeophtheirus salmonis*) was carried out and the effects were evaluated in the next generation. Hatching success and phenotypical abnormality in the larvae was investigated. Q-PCR was performed with RNA from the mother louse to address the knock down efficiency of the hormone receptor following the RNAi experiment.
Hormone receptors do not necessarily have to be highly expressed as they are switches in the beginning of gene cascades. Therefore it is not astonishing that there could be seen very big effects in the development of the larvae even though little down regulation was found. Two different analytical methods (the ΔΔCt method and a kinetic approach) were compared and the results were discussed serving as an example of the importance of choosing the appropriate methods when evaluating Q-PCR data.

P088

Investigation of the potential transfer of recombinant DNA from feed into milk of cows fed genetically modified maize

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Despite the consecutive increase in global adoption of genetically modified (GM) crops, there is an ongoing debate about potential effects and the fate of recombinant DNA in the food derived from animals fed GM crops. To address the food safety concerns of the public regarding the potential transfer of recombinant DNA (*cry1Ab*) into the milk of cows fed GM maize (MON810), a highly specific and sensitive qPCR assay was developed for monitoring suspicious presence of novel DNA in bovine milk. The developed assay was validated according to the “Minimum Performance Requirements for Analytical Methods of GMO Testing” published by the European Network of GMO Laboratories (ENGL).
36 lactating Simmental cows, housed at the Bavarian State Research Center (LfL, Grub, Germany), were fed on GM maize (MON810) or the non-transgenic counterpart for 25 months. Milk samples were taken monthwise and analyzed for the presence or absence of *cry1Ab* DNA.

DNA from whole milk was isolated following the optimized procedures published by the Federal Office of Public Health (FOPH, Bern, Switzerland) and analyzed by amplification of a 206bp fragment of *cry1Ab* , a 354bp fragment of *GAPDH* and a 173bp fragment of *rubisco* by means of qualitative endpoint PCR. For qPCR, the LightCycler-system (Roche) with SYBR green was applied for the quantification of a 206bp fragment of *cry1Ab* . The data obtained were analyzed using the standard curve method. To generate a standard curve, milk samples were spiked with genomic DNA from GM maize (MON810) containing different copy numbers of *cry1Ab* (10 to 106) and the DNA was reisolated following the FOPH protocol. To specify the efficiency of the optimized extraction method, the recovery rate was determined by re-isolation and quantification of *cry1Ab* in milk samples spiked with three different copy numbers. Furthermore, inter- and intra-assay coefficients of variation (CV) were verified by analysis of three different samples within the standard curve.
The detection limit of the qPCR was 100 copies of *cry1Ab* per µL milk. A mean recovery rate of 84.9% (n=3, six replicates each), an intra-assay CV of 0.15 (n=9) and an inter-assay CV of 0,78 (n=9, three replicates each) illustrate the suitability of the extraction and quantification procedure for novel DNA in whole milk. Using this assay for milk samples collected from cows fed either transgenic or non-transgenic rations for 25 months, fragments of *cry1Ab* were not detected in the analyzed samples at the assay detection limit.

P089

Real-time PCR assays based on the multi-copy rDNA ITS region and the single-copy β-tubulin gene for detection and quantification of the strawberry pathogen Colletotrichum acutatum

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C. acutatum is one of the most important fungal pathogens of strawberry worldwide. The disease is responsible for up to 80% plant death in nurseries and yield losses of over 50% in strawberry production fields. Symptoms include fruit rot, crown rot and lesions on stolons. However, *C. acutatum* may also persist on young strawberry plants without causing visible symptoms. Such latent infections are considered to be the main cause of dissemination of *C. acutatum* . Detection and quantification of *C. acutatum* during this latent phase using real-time PCR might aid considerably in the reduction of its spread. This paper describes the development of real-time PCR assays using primers and probes designed to the multi-copy rDNA ITS1 region and the single-copy β-tubulin 2 gene of *C. acutatum* . The sensitivity of both assays is compared and the data are used to calculate the genome size and ITS copy number of *C. acutatum* . Finally, detection and quantification of *C. acutatum* is demonstrated in artificially and naturally infected strawberry leaves.
Using TaqMan technology, the ITS-based real-time PCR assay could reliably detect as little as 50 fg genomic DNA, 100 copies target DNA, or 25 conidia. The β-tubulin-based assay was circa 66 times less sensitive, and therefore less suitable for detection purposes. However, by applying the β-tubulin primers together with the ITS primers to both genomic DNA and known numbers of cloned target DNA, they proved very useful in revealing some insights into the genome of *C. acutatum* . Specifically, we estimated a genome size of 60.0 Mbp for *C. acutatum* and the presence of circa 20 tandem copies of the ITS region in one genome of *C. acutatum* . Concerning the detection of *C. acutatum* in strawberry leaves, we were able to detect *C. acutatum* in plant tissue mixes containing only 0.001% infected tissue. In addition, real-time PCR analysis of leaf samples taken at various times after inoculation indicated that the assay allows monitoring of early symptomless growth of *C. acutatum* on strawberry leaves. Finally, the assay allowed detection of *C. acutatum* in naturally infected and symptomless strawberry leaves collected from production fields and planting material.
In conclusion, our results demonstrate that the ITS-based real-time PCR assay developed in the present study, is a highly sensitive technique that can be used in routine quarantine inspections to screen strawberry planting material for *C. acutatum* contamination.

P090

The use of TaqMan qPCR to detect poultry ingredient in white fish surimi

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Surimi, a minced and washed fish muscle products consisting of salt soluble-myofibrillar proteins, is used as an functional ingredient in seafood analogs. The most common fish used in the production of surimi is Alaska Pollock or white fish (*Theragra chalcogramma*). One reason white fish is so widely used in the production of surimi, besides its abundance, is the yield of cohesive gels created during processing, which is essential for the creation of the final product.
Surimi is mainly produced in the Far-East as a source of alternative fish protein. In produce of this sort there is always a chance of proteins other than fish, being incorporated such as proteins of poultry origin with the danger of introducing poultry that was exposed to the highly pathogenic avian influenza virus.
In an effort to develop a system that will enable detection of proteins of poultry origin in imported surimi, a TaqMan real-time qPCR reaction which was specifically designed to detect chicken mitochondrial cytochrome c sequences, was implemented.
To examine the reaction specificity, white fish, chicken embryo, chicken breast, pig kidney tissue culture cells and cattle mitochondrial DNAs were tested.
Only mitochondrial DNAs extracted from chicken embryo and breast, reacted positively, contrary to negative reactions when mitochondrial DNAs from white fish, pig kidney tissue culture cells and cattle meat were used.
To test the presence of materials of poultry origin in surimi, mitochondrial DNA from surimi was extracted and served as template in the reaction. Positive reaction in the surimi samples indicating the presence of chicken origin materials could be demonstrated.

P091

UTILIZATION OF FTA (R) CARDS COMBINED WITH ONE-STEP REAL-TIME PCR FOR RAPID DETECTION OF QUARANTINE VIRUSES AND PHYTOPLASMAS

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Smooth international trade of planting material often depends on efficient inspections at the port of entry. Therefore, reliable and fast screening for regulated plant pathogens is an important challenge. This study evaluates the use of Whatman FTA (R) card technology (a commercially available filter paper impregnated with patented chemicals) as RNA and DNA extraction method compared to commercial plant extraction kits (DNeasy, RNeasy Plant – Qiagen; Invisorb DNA and RNA Plant – Invitek), combined by a one-step qPCR detection for a selection of regulated pathogens in host plants. The method was tested for 2 viruses (*Pepino mosaic virus* , Potexvirus, Flexiviridae; *Tomato spotted wilt virus* , Tospovirus, Bunyavirudae) and a phytoplasma (*Apple proliferation phytoplasm* , AP Phytoplasma). A dilution series of respective *in vitro* transcripts of the target RNA fragment served as standard for quantification of the RNA viruses in the infected leaf samples. Quantification of phytoplasma DNA was done by means of plasmids containing a 16S rDNA target fragment. Both were added to a mock-RNA/DNA extraction of healthy control plants. With the commercial RNA and DNA extraction kits detection was more sensitive and quantification more reliable than with the FTA card method. However, the FTA card technology followed by a one-step qPCR detection enabled reliable screening of leaf tissue for infection with our target regulated pathogens in less than 4 hours. For the viruses, the RNA fixation on the filter paper was most efficient when the leaf samples were homogenised in a standard ELISA extraction buffer. For AP Phytoplasma DNA, a direct leaf punch on the FTA cards was sufficient for a reliable detection of this pathogen in infected leaf tissue. In conclusion we can state that the FTA card technology followed by one-step qPCR could offer a fast and reliable screening method for sanitary control on the regulated pathogens.

P092

Quantitative Characterization of the Most Relevant Prostaglandins in the Bovine Uterus: Embryonic Impact generates an Intrauterine Environment of Prostacyclin and Prostaglandin PGF2alpha outranging PGE2 by far

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The establishment of pregnancy is dependent on an intact embryo-maternal communication especially prior to implantation. Prostaglandins (PG) are important regulators of several reproductive processes. We analyzed the most relevant PG in bovine uteri at different preimplantation pregnancy stages as compared to non-pregnant controls. Additionally, endometrium and trophoblast tissue samples were examined regarding the expression of specific enzymes and receptors involved in PG generation and function. Simmental heifers were artificially inseminated (pregnancy) or received seminal plasma only (controls). At days 12, 15 or 18 post estrus uteri were flushed with 100mL PBS for PG determination by LC-MS/MS. Endometrial and trophoblast tissue was sampled for RNA extraction and RT-qPCR analysis. At all days and time points examined, the concentration of 6-keto-PGF1alpha (PGI2-metabolite) was predominant followed by PGF2alpha > PGE2 > PGD2 = TXB2. At days 15 and 18 all PG increased from low levels at day 12, with a much higher increase during pregnancy. The highest PG concentration was measured at day 15 of pregnancy with 6-ketoPGF1alpha (6.4 ng/mL) followed by PGF2alpha (1.1 ng/mL) and PGE2 (0.3 ng/mL). The ratio of PGE2/PGF2alha was neither influenced by day (P=0.09) nor status (P=0.4). The PG contribution of the embryo was high as seen from abundant endometrial PG synthase transcripts together with low catabolic enzyme expression and evidenced by higher levels of PG than respective metabolites. While the endometrium expressed more PGE2 receptor (EP2) than the embryo, the embryo revealed more transcripts for PGF2alpha receptor (FP) than the endometrium. This study provides comprehensive quantitative insights into the dynamic regulation of PG of the bovine uterine lumen in vivo. Next to PGE2 other PG, namely PGI2 and PGF2alpha may have an important role for the developing embryo and thus may be essential rather than detrimental for successful reproduction.
Supported by DFG (UI 350/1-2, FOR 478)

Diagnostic & Molecular Markers (human)
Location: poster room (mensa)

P093

IDENTIFICATION AND QUANTIFICATION OF THE RARE BCR-ABL TRANSCRIPTS e13a3 AND e1a3.

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OBJECTIVE: The vast majority of cases of chronic myelogenous leukemia, and a minority of acute lymphoblastic leukemia cases, are associated with the presence of the Philadelphia-chromosome. This represents a balanced translocation fusing the c-ABL gene of chromosome 9 to the BCR-gene of chromosome 22. Most common isoforms of BCR-ABL are e13a2 and e1a2, both with fusion of BCR to ABL exon 2 We, in this poster, describe the detection of two rare BCR-ABL fusion gene transcripts, and the establishment of the quantitative analysis for these transcripts.
METHODOLOGY: Peripheral blood samples from two patients were referred to our routine laboratory for detection and eventual identification of BCR-ABL fusion gene transcripts. The first case is a 59 years old woman with acute lymphoblastic leukemia (ALL). The second case is a 26 years old woman diagnosed with chronic myeloid leukemia (CML).
For determination of BCR-ABL fusion gene transcripts, two different PCR strategies were used. One was a qualitative analysis making use of conventional multiplex PCR. Agarose gel electrophoresis of the PCR-products was performed. Simultaneously a real time quantitative RT-PCR analysis (RQ-PCR) was conducted with accordance to the recommendation of the EAC consortium. The amplicons of interest were sequenced, and the selected primers and probes were tested for a Taqman based RQ-PCR. Cloning of the patient sample with TOPO TA cloning kit was done to produce plasmids for the generation of standard curves.
RESULTS: The amplified products of the multiplex PCR showed bands with unexpected sizes for both of the patients. The PCR product from the patient with ALL had a size of about 230 bp and that of the CML patient about 260 bp. The sequencing results showed that the PCR product of the ALL patient had an exact length of 235 bp, and identified the transcript as a product of an e1a3 fusion gene. Similarly, sequencing of the PCR product from the CML patient identified a segment of 266 base pairs. The transcript was a product of an e13a3 fusion. By combining the EAC forward primers used for detection of e13/14a2 and e1a2 transcript with the ABL control gene probe and the reverse primer which are located in exon 3, a RQ-PCR analysis could be performed. Quantitation of e13a3 was done at diagnosis and after the patient had undergone treatment. After 3 months on standard treatment (Imatinib mesylate 400 mg once daily) our RQ-PCR showed a 2-log reduction of BCR-ABL. Quantification of e1a3 after treatment was not possible as the patient died short time after initiation of therapy.
CONCLUSION: Detection of the BCR-ABL transcripts lacking exon 2 in our multiplex PCR, was made possible due to the location of the reverse primer in exon 3 of the ABL-gene. We describe the establishing of a real time quantitative PCR analysis, based on the EAC protocol, which allows the quantification of diagnosis and follow-up samples of the rare transcripts e13a3 and e1a3.

P094

Somatostatin and dopamine receptor expression influences the effects of selective ligands on cell viability in non functioning pituitary adenomas in vitro

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Previous studies demonstrated that drugs interacting with dopamine (DR) and somatostatin receptors (SSTR) might be useful in non-functioning pituitary adenoma (NFPA) medical therapy. The aim of our study is to evaluate the effects on cell viability of the SSTR/DR ligands BIM23A370 and BIM23A387 (SSTR2>DRD2) in 6 NFPAs in vitro (#1 - #6) and BIM23A760 (SSTR2>>DRD2>SSTR5) in 8 NFPAs in vitro (#7 - #14), investigating SSTR (subtypes 2 and 5) and DR (subtype 2) expression pattern. Primary culture cell viability is assessed after a 48 h treatment with each compound at 10 nM by a colorimetric method. Since the normal counterpart is not available, Absolute Quantitative Real Time PCR method is used to quantify SSTR2, SSTR5, and DRD2 expression, with a 6 points standard curve (range: 10^3-10^8 molecules of target cDNA). To normalize the results, each sample is quantified for housekeeping gene 18S expression. All the data are expressed as target cDNA molecule number/1 µg of retrotranscribed total RNA. SSTR2 is expressed in 10 NFPAs (71.4 %), at variable levels (6000-720000). SSTR5 is poorly expressed (10681) in only 1 NFPA (7.1%), while DRD2 is highly expressed (51000-2721320) in 9 NFPAs (64.3%). In samples #1, #4, and #6 expressing SSTR2 but not DRD2, treatment with BIM23A370 or BIM23A387 increases cell viability (15-360%, $p<0.01$). In sample #2, expressing DRD2 (51080) but not SSTR2, treatment with BIM23A387 induces a 25% ($p<0.01$) decrease of cell viability, while treatment with BIM23A370 doesn't influence cell viability. In sample #3, expressing all 3 receptors (SSTR2:144208; SSTR5:10681; DRD2:229926), treatment with BIM23A370 causes a 12 % ($p<0.01$) reduction in cell viability, while no change is recorded after treatment with BIM23A387. In sample #5, lacking the expression of all 3 receptors, treatment with the chimeric molecules results in a 20 % ($p<0.01$) increase in cell viability. Treatment with BIM23A760 does not influence cell viability in samples #7 - #14, except in sample #8, expressing DRD2 (73494) and SSTR2 (114705), where a reduction in cell viability (-15 %, $p<0.01$) is evident. It might be relevant that this sample expresses SSTR2 at higher levels than all other NFPAs.

SSTR/DR ligand BIM23A370 causes a significant reduction in cell viability only in NFPA with both SSTR2 and DRD2 receptors reaching an expression level >100000 and >200000, respectively. In all other samples, treatment with this chimeric molecule leads to an increase in cell viability. Treatment with receptor ligand BIM23A387 causes a reduction in cell viability in samples expressing DRD2 <100000, while it has no effect in samples expressing DRD2 >100000. SSTR/DR ligand BIM23A760 reduces cell viability only in one case showing DRD2 expression<100000 and SSTR2 expression>100000. In conclusion, these molecules could be useful in NFPAs medical therapy, but further investigations are necessary to assess SSTR/DR expression pattern in order to maximize effects on cell viability.

P095

VAP (Ventilator-Associated Pneumonia) DIAGNOSIS USING MOLECULAR MARKERS. PRELIMINARY RESULTS.

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VAP (Ventilator-Associated Pneumonia) is one of the most common infectious complications in intensive care units as a consequence of intubation and mechanical ventilation support. Early-VAP appears during the first four days of mechanical ventilation while Late-VAP develops ≥ five days after. Taking into account that about half of episodes of VAP appear, within the first four days of intubation, the possibility of detecting the cited increase few days before the apparition of the symptoms give us the chance of avoid the progression of the infection. According with previous reports using micro array technique several genes related with immune activity and immune response to bacteria or infection, were tested in order to establish the best marker to the early detection of VAP. Objectives : Determination of the appropriate marker for the early diagnose of VAP in Intensive Care Unit patients mechanically ventilated in order to avoid the infection progress. Methods : Twelve patients (10 men-2 women; mean age 57.58) were included in the preliminary study after being mechanically ventilated. Patients who develop VAP were included in the problem group. Arterial blood samples were obtained each 72 hours since intubation day, considered the initial sampling point. Samples were processed to extract RNA from leukocyte fraction, cDNA was then synthesised using random primers. Afterwards, several genes related with infectious response were tested by Real time PCR: Lactotransferrine (LTF), Cathelicidin Antimicrobial Peptide (CAMP), Phospholipid Scramblase 1 (PLSCR1), Inhibin Beta A (INHBA) and Hydroxyprostaglandin Dehydrogenase 15-NAD (HPGD). Results : After performing relative quantitation at different time points using as reference gene β Actine, a marked increase in HPGD expression (ratio=10.23) were observed in the problem group versus control at point 3, on the other hand the ratio at point 2 (before the apparition of the symptoms) is higher in control group than in problem group.
Conclusion : HPGD gene seems to be the most adequate marker for the early diagnose of VAP in ICU patients with mechanic ventilation. Currently, the aim of the present project is to determine the adequate time point where the expression increase appears.

P096

Novel Protein Expression Assays using qPCR for the Detection and Relative Quantification of Protein Markers in Human Embryonic Stem Cells

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Quantitative PCR (qPCR) has revolutionized the characterization of nucleic acids in cells, and several classes of cellular nucleic acids are routinely analyzed by qPCR assays, including genomic DNA, mRNA and microRNAs. Proximity ligation assay (PLA) technology extends qPCR applications now to the detection of cellular proteins through the amplification of a surrogate DNA template. PLA is a three-step process that involves, 1) binding of paired antibody-oligonucleotide probes to a protein target in biological samples, 2) templated ligation of the oligonucleotides in proximity, and 3) qPCR detection. We have optimized this technique for crude cell lysates utilizing a simple, one-step sample lysis approach to release all classes of proteins, and combined it with gold standard TaqMan® chemistry to create a highly sensitive and specific process for measuring protein expression in small samples. One application of this assay is the detection and relative quantification of markers in pluripotent and differentiated stem cells. Stem cell characterization typically relies on determining the presence and amount of stage specific protein markers such as OCT4, NANOG, SOX2, and LIN28. Protein expression results confirm cell-stage specific changes in protein expression of these key stem cell markers, and the data can be directly compared with published mRNA expression profiles for the same cell lines. We have engaged a number of researchers as test sites for these assays and are gathering input and feedback. Our findings illustrate how this new assay system expands the scope of qPCR to protein detection and quantification, an important area of cell biology.

P097

The developmental changes in mitochondrial DNA content and expression levels of genes involved in pathway from PGC1 to mtDNA replication in human tissues

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The inadequate efficiency of mitochondrial biogenesis leads to low energy production which may play a crucial role both in the fetal development and neonatal morbidity. Therefore we focused our work on finding the first markers of mitochondrial proliferation on transcriptional level during human prenatal development. The aim of our study was to characterize the changes in expression of genes involved in the regulation and maintenance of mitochondrial DNA content (peroxisome proliferatoractivated receptor-g coactivator-1a, PGC1; nuclear respiratory factor 1, NFR1; mitochondrial transcription factor A TFAM; polymerase gamma – catalytic subunit, POLG). Presently we analyzed both mtDNA amount and expression levels of chosen genes in human fetal tissues during gestation. DNA and RNA were isolated from 26 pairs of liver and muscle tissue samples obtained at autopsy from miscarriages after informed consent, between 13 th and 28th week of gestation. The mtDNA amount and gene expression levels were analyzed by the real-time PCR method using SybrGreen I. In both fetal liver and muscle tissues, mtDNA content and TFAM expression levels were increasing with onward fetal development (mtDNA: $r = 0,50$; $p < 0,01$; respectively $r = 0,62$; $p < 0,01$); (TFAM: $r = 0,56$; $p < 0,01$; respectively $r = 0,61$; $p < 0,01$). On the other hand, POLG expression level was increasing only in fetal liver tissue ($r = 0,54$; $p < 0,01$). NRF1 was unchanged in both fetal tissues and PGC1 was slightly rising between 13 th and 28th week of gestation in liver tissue ($r = 0,42$; $p < 0,05$). Our results showed that POLG expression varies between two different tissues during gestation and probably is not associated with mtDNA content as tightly as TFAM expression. The increase of PGC1 transcript level is statistically significant in liver tissue and we suggest that it could bear evidence of tissue specific expression or regulation. Therefore the binding of NRF1 to its specific sites which are situated on the promoter of TFAM is positively affected by increasing expression of PGC1 in liver tissue.
In conclusion, this study described mainly the increasing trends of gene expression in the pathway leading from expression of PGC1 to mtDNA content changes in fetal liver and muscle tissues during second trimester of gestation. Our study involved the largest set of human fetal tissue samples as it has been used for such study to date. According to our results, we suppose that the mitochondrial proliferation is growing up between 13 th and 28th week of gestation. Therewithal it is the first hallmark of prepare for postnatal adaptation which is evident on transcriptional level in this fetal period.
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P098

Target-assembled in-situ detection by DNA-mounted exciplexes, heteroexcimers and excimers based on 2-arylalkynyl-pyrene.

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Target-assembled in-situ detection by DNA-mounted exciplexes, heteroexcimers and excimers based on 2-arylalkynyl-pyrene.
Reversible hybridisation of complementary polynucleotides is essential to the biological processes of replication, transcription, and translation. Physical studies of nucleic acid hybridisation are required for understanding these biological processes at a molecular level. In addition the physical characterisation of nucleic acid hybridisation is essential for predicting the performance of nucleic acids in vitro, for instance, in hybridisation assays used to detect specific polynucleotide sequences.
In the recently discovered split-oligonucleotide (tandem) probe system for exciplex-based fluorescence detection of DNA, the detection system is split at a molecular level into signal-silent components. For fluorescence emission by the exciplex-forming system of two short probe oligonucleotides and their target nucleic acid, the components must be assembled correctly and very precisely into a specific 3-dimensional structure to ensure close proximity of the exciplex partners. The study reports the effects of structural variation of the exciplex partners to make use of the spectroscopic properties of alkynyl-substituted pyrenes as exciplex partners, a structural change which allows excitation to occur in the visible region. The exciplexes formed emitted at 505 nm (broad bands) with large Stokes shifts (>100 nm). The effects of linker length and relative locations of the exciplex partners were assessed for target sequences based on (i) a region of the Leishmania genome and (ii) the region corresponding to the cytochrome P450 CYP2C9 3*allele (wild type and SNP). The molecular nature of the gap region in the tandem nucleotide system constructed for such tandem duplexes was studied using melting temperatures based on both the absorbance at 260 nm and on the fluorescence of the exciplexes formed, as well as by the efficiency of the exciplex formed by such systems, the exciplex providing a new and extremely sensitive probe of subtle structural and conformational manifestations in DNA duplexes. Exciplex structural variation provides a broader range of structural insight than can be expected from analogous systems based on excimers for which the choice of partners is very limited.

P099

Nucleic acid diagnostic tests for the detection of microbial pathogens

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The Molecular Diagnostics Research Group (MDRG) at NUI Galway has over 20 years experience in the field of molecular diagnostics. Research activities include molecular diagnostic target discovery and nucleic acid based test design and development. Over the years the group has identified a number of platform nucleic acid diagnostic test (NAT) target technologies for the sensitive and specific detection and identification of bacterial and fungal pathogens. The NAT target technologies have been demonstrated in a wide range of test formats including, real-time PCR, real-time NASBA and direct nucleic acid target detection systems. Applications of the NAT technology include clinical, food, veterinary and environmental sectors. Successful commercialisation of our nucleic acid targets has occurred through licensing agreements and research and development collaborations with many of the major international diagnostics companies. Detailed below are some of the strategies and methodologies employed by the MDRG in the design and development of NATs for the detection and identification of micro-organisms.

P100

D1 – D5 dopamine receptors expression in paranoid schizophrenia patients

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Schizophrenia, commonly developed in young adults that is one of most common mental disorders, but the phatophysiology and etiology conditions of schizophrenia is still obscure. Based on Numerous studies about dopamine and schizophrenia, it suggested that changes in the dopamine systems are in related with schizophrenia, but still there is no clear direct evidence for dopamine hypothesis in schizophrenia.
In terminated examination, 30 paranoid schizophrenia patients mRNA from white blood cells extracted , then cDNA were synthesized . After Quantitive Real-time PCR examination with the related primaries for D1 – D5 receptors were terminated and the compared consequences in abundance of genes expression with the normal samples reveals that D1 – D5 dopamine receptors were expressed in all samples . Abundance of normal individuals were D1 100% , D2 16.6 % , D3 40 % , D4 83.3 % , D5 86.6 % and for patients were D1 100% , D2 83.3 % , D3 30 % , D4 83.3 % , D5 83.3 % .

P101

Gene expression profiling discriminates biopsies in paediatric patients investigated for suspected celiac disease

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Background and aim Celiac disease (CD) is an inflammatory disease, triggered by an immunologic response to gluten in wheat, which manifests itself by symptoms such as diarrhoea, abdominal pain and malabsorption. CD results in an inflamed small intestine with varying degree of villous atrophy, crypt hyperplasia and increased paracellular permeability of the intestinal epithelium. According to current guidelines, the diagnosis of CD requires intestinal biopsy and is set by histopathological findings. The aim of this study was to investigate the possibility of establishing molecular classifiers using gene expression data.
Materials and methods Unselected paediatric patients investigated for suspected CD were consecutively included in the study, and total RNA was isolated from small intestinal biopsy samples. Using real-time PCR and relative quantification, gene expression was investigated in biopsies from four healthy individuals, ten with CD (histopathologically Marsh 1 – 3C) and one biopsy obtained after introduction of gluten-free diet. A total of 33 genes were analysed: five villi markers, 11 crypt markers and 17 apical junctional complex (AJC) genes. Expression data was normalized using two reference genes exhibiting low sample-to-sample variation (M-value of 0.19 according to the geNorm algorithm), and statistically investigated for differential expression using Mann-Whitney ($p < 0.05$, uncorrected for multiple testing). Sample classification based on gene expression profiling was explored by means of principal component analysis (PCA) and hierarchical clustering. The study was conducted under approval by the regional ethics committee.
Results Sixteen of the investigated genes were differentially expressed (healthy vs. Marsh grade 2-3): all of the villi markers, three crypt markers and eight AJC genes. All of the differentially expressed genes except two of the crypt markers (0.63- and 0.72-fold in healthy compared to Marsh grade 3) exhibited higher expression (1.3 – 3.5-fold) in healthy biopsies. Based on the degree of intestinal mucosal lesion classified according to Marsh criteria, PCA and cluster analysis resulted in the following three relevant groups: healthy / Marsh type 0 / Marsh type 1, Marsh type 2, and Marsh type 3.
Conclusions Preliminary data indicate that gene expression profiling of intestinal biopsies is a feasible approach to classification and that this may prove useful in confirming the diagnosis.

P102

Identification of Bacillus anthracis by a specific chromosomal marker

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Correct identification of *Bacillus (B.) anthracis* and distinguishing this organism from closely related *B. cereus* and *B. thuringiensis* is still one of the big challenges in *B. anthracis* diagnostics. The differentiation between non-anthracis *Bacillus* -species harbouring anthrax-specific virulence plasmids, plasmidless and plasmid-harbouring *B. anthracis* strains is one of the major problems of commercially available PCR-kits. Primer and probes for amplification of chromosomal markers are either not present or not specific. Many markers have been describedto be highly specific, but overtime, several exceptions for these markers became public. Today, analysis of published genomes reveals possible marker genes to be more specific for *B. anthracis* , like the locus BA_5345. In this study we designed a TaqMan® PCR targeting sequence of the latter gene. A panel of 328 *Bacillus* species was used to determine specificity. Probit analysis was performed to ascertain sensitivity. All *B. anthracis* isolates (n=92) were specifically detected by using the genomic TaqMan® PCR assay whereas 236 strains belonging to 19 *Bacillus* species other than *B. anthracis* showed negative results. The detection limit was calculated to be 12.7 copies per reaction (95% confidence interval, 10.2 – 17.5 copies). Here we present a well-evaluated and actually highly specific TaqMan® PCR assay for the unequivocal detection of *B. anthracis* based on a chromosomal marker.

P103

Quantitative real-time PCR measurement of NPM1A Mutations following Allogeneic Stem Cell Transplantation in Patients with Acute Myeloid Leukemia

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Background: Following allogeneic stem cell transplantation, minimal residual disease (MRD) diagnostics in acute myeloid leukemia (AML) provides an option for early detection of relapse. The Nucleophosmin (NPM1) mutations represent the so far most frequent known molecular marker in AML with a specific association to normal karyotype AML. Most frequent is the NPM1 subtype consisting of a four base pair insertion. We here evaluated the utility of this marker for follow-up in the post-transplant period in AML. Patients and Methods: A cohort of 13 mutated AML patients receiving 14 allogenetic stem cell transplantations (SCT) from related or unrelated donors was retrospectively screened by quantitative real-time PCR (qPCR) for NPM1A mutations on bone marrow or peripheral blood samples which had been cryopreserved before and after SCT. Normalization was performed by quantification of the HCK gene. Median follow-up was 7 months (range, 1 – 60 months) from SCT. Results: After SCT, 10 of 14 mutated transplantation cases (71%) became PCR-negative. Four of those achieved stable remission. In contrast, all four cases (29%) remaining NPM1Amut positive post-transplant developed morphological relapse, in all being accompanied/preceded by increase of the NPM1A/HCK mutation level. Increase of NPM1mut was seen earlier than morphological relapse with a mean interval of 24 days (range, 12-38 days). Conclusions: According to this series, quantitative assessment of NPM1mut seems suitable to follow MRD in the post-transplant period and can indicate relapse earlier than morphology. This might allow an earlier start of adoptive immunotherapy in case of impending relapse.

P104

Bladder cancer detection with survivin mRNA as a molecular marker

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Objective - The presented investigation was carried out in patients with suspected bladder tumors to evaluate the feasibility and discrimination characteristics of survivin mRNA measurement as a diagnostic and potential prognostic molecular marker for transitional cell carcinoma (TCC). Aim of the study was to reveal possible influencing factors and estimate a cut-off for the discrimination between positive and negative diagnostic findings.
Methods - Survivin was measured by an mRNA assay with anonymized cell samples isolated from voided urine. The specimens were taken from 50 patients with the suspicion of new or recurrent TCC prior to transurethral resection. The study group consisted of 33 male and 17 female patients. Mean age was 68 years. Histopathological evaluation revealed no malignancy in 18 (36%) patients and confirmed TCC in 32 (64%) patients (9 pTaG1, 11 pTaG2, 4 pT1 G2-3, 4 > pT2, 4 pCis). To preserve the samples, cell pellets of centrifuged urine samples were frozen in buffer solution. These samples were shipped to BGFA laboratory, where RNA was isolated and reverse transcribed, before survivin mRNA was amplified by nested PCR and quantitated by Real-time PCR. ß-Actin mRNA was determined for sample quality control. Furthermore ROC analyses for the survivin assay were performed considering the influence of hematuria, cystitis, and other urological diseases.
Results - The determination of ß-actin showed that 49 (98%) of the samples had sufficient integrity for the mRNA assay after freezing and shipping. The ROC analysis identified a cut-off level of 10,000 mRNA copies of survivin, resulting in a sensitivity of 53% and a specificity of 89%. Six out of the 20 pTa tumors (30%), all pT1 and all invasive tumors were detected. Of the 4 patients with pCis 3 (75%) could be identified. Only two patients (4%) were assessed as false positive. One of them suffered from urolithiasis and the other is under ongoing control because of positive cytology findings. The different variables as diseases, age, and gender did not significantly influence the marker levels.
Conclusion - Survivin might be a suitable diagnostic marker for bladder cancer. In this study, we found no confounding factors of survivin mRNA levels but the statistical power was limited. The specificity (89%) was very promising especially considering the clinical information about the false positive patients. Sensitivity in high-grade tumors was excellent (91%), however, the lower sensitivity on low-grade tumors needs to be evaluated in large prospective studies to assess survivin as screening marker.

P105

REAL-TIME PCR ANALYSIS OF NNMT EXPRESSION IN URINE AND TUMOR TISSUE OF BLADDER CANCER PATIENTS AND ITS POTENTIAL RELEVANCE FOR DISEASE DETECTION.

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Carcinoma of urinary bladder ranks among the top ten most common cancers worldwide and the majority of these tumours recur and progress into muscle-invasive disease, thus needing for long-term and frequent follow-up. Despite its invasive nature, cystoscopy remains the gold standard diagnostic evaluation for detection and surveillance of bladder cancer. Urinary cytology is used as an adjunct to this procedure, but lacks sensitivity for low-grade tumours. More sensitive and non invasive methods are therefore required, what fosters continuing interest in identifying new bladder cancer markers.
To explore the involvement of enzymes of drug transport, phase I metabolism, and phase II metabolism in bladder cancer, in the present study we analysed the gene expression profiles of tumour and non-tumour tissues obtained from the same patient by DNA macroarray. The enzyme Nicotinamide N-methyltransferase (NNMT) 1 was identified as a highly expressed gene in bladder cancer. Real-Time PCR, Western blot analysis, and catalytic activity assay performed on a large cohort of patients with transitional cell carcinoma (TCC) confirmed NNMT upregulation. Differential specific activity measurements (tumour versus adjacent normal tissue/ carcinoma in situ) revealed NNMT overexpression in all TCCs, with fold change values ranging between 1.3 and 383.2 (mean 49.4-fold).

NNMT mRNA levels were also determined in urine specimens obtained from 20 patients with bladder TCC and 20 healthy subjects. We found that NNMT expression levels were significantly higher in patients with bladder tumour compared to controls, that showed very low or undetectable amounts of NNMT transcript.
Our experimental data indicate that a marked NNMT increase is a peculiar feature of TCC and suggest the potential suitability of urine NNMT expression levels determination for the diagnosis of bladder cancer.
1. Sartini D et al. (2006) J Urol 176(5): 248-54.

P106

Analysis of the Survival motor neuron gene promoter: effects of histone deacetylase inhibitors on histone acetylation, expression, and DNA methylation
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Spinal muscular atrophy (SMA) is caused by defects in Survival motor neuron (SMN) gene. Two SMN genes exist in human genome, a telomeric SMN1 and a centromeric SMN2. Despite their similarity in coding sequence, only SMN1 produces sufficient levels of a full-length mRNA transcript, while SMN2 is spliced by exon-skipping into non-functional deletion variant. In SMA patients SMN1 is disrupted or lost. Recent studies show a partial improvement of clinical state of SMA patients upon administration of histone deacetylase (HDAC) inhibitors. To investigate molecular principles underlying the therapeutic effect of HDAC inhibitors we examine: i) the effect of HDAC inhibitors (VPA and M344) on histone acetylation of the SMN promoter and SMN2 gene expression; ii) the effect of HDAC inhibitors on BCL2 expression; and iii) DNA methylation status of SMN promoter and its changes in response to VPA treatment. Our results demonstrate, that M344 is more efficient HDAC inhibitor compared to VPA, but the histone hyperacetylation induced by either of these inhibitors is not sufficient to increase substanially SMN2 expression, or to shift its splicing pattern towards a full-length variant. However, up to 4-fold increase was observed in expression of the anti-apoptotic gene BCL2 after VPA treatment. Further, we evaluated DNA methylation status and DNA methylation changes in the SMN2 promoter region after VPA treatment. The decrease in methylated CpGs was 6% (from 55 to 49%), thus showing relatively weak demethylating effect of VPA, at least in this particular region.
The work was supported by the grants of MSMT LC06023 and MSM0021622415.

P107

Gene Expression Profile of hOGG1 and its Splice Variants in Human Skin Samples and Cell Cultures
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Human 8-oxoguanine DNA glycosylase 1 (hOGG1) is the most important base-excision repair enzyme to prevent mutations by 7,8-dihydro-8-oxoguanine (8-oxoG), the major DNA damage caused by reactive oxygen species (ROS). Eight different isoforms caused by alternatively spliced mRNAs of hOGG1 are described and classified in two main groups (type 1 and type 2) depending on the last exon of their mRNA sequence. The characteristics of any of these isoforms have not yet been determined, but type 1a (targeted to the nucleus) and type 2a (targeted to mitochondria) were identified as the two main isoforms in several human tissues. In skin hOGG1 plays an important role in preventing pathological processes like skin aging and carcinogenesis caused by UV-induced ROS. Despite its crucial role it has not yet been examined which splice variants of hOGG1 are most prominent in human skin cells and how they are regulated. In this study we determined the expression of hOGG1 and its splice variants in human skin samples and cultured fibroblasts, keratinocytes and melanocytes. Therefore we designed primer pairs for every hOGG1 splice variant by using different exon combinations respectively exon-exon transitions to determine the expression of the splice variants separately. For the relative quantification of hOGG1 and its splice variants SYBR-Green qRT-PCR was used. The normalization was carried out against either β-Actin to determine the overall expression of hOGGI and its correlation to the expression of single splice variants in detail, as well as against hOGG1 itself to estimate the contribution of the splice variants to the overall expression. The analyzed cell types showed no significant differences concerning their splicing patterns. Isoform 1b was most expressed, followed by 2e before 2a and 1c. The isoforms 2b, 2d, 2c and 1a showed only low expression levels. Thereby splice variants 1b, 2d, 2e, 2a and 2c showed a good correlation (R^2 = 0.6 -0.9) with hOGG1 expression levels. The splice variants 1a, 1c and 2b were not correlated with hOGG1 expression (R^2 = 0.05 - 0.2). Also we could identify a very weak expressed additional hOGG1 splice variant by RT-PCR and subsequent cloning. The high expression level of 1b has not yet been detected in other tissues and might indicate a special role of this isoform in human skin cells. Also the full length nature of hOGG1 1b has not yet been analyzed. It is putatively targeted to mitochondria, may be the major mitochondrial targeted hOGG1 isoform in skin and features therefore a potential suitability as biomarker for mitochondrial DNA repair. Further experiments are planned to confirm these results by testing hOGG1 gene expression in other tissues and cell types with known splicing patterns and by overexpression / silencing experiments of single hOGG1 isoforms.

P108

Quantitative real-time PCR for the detection of Streptococcus pneumoniae from the Viridans group streptococci targeting the cpsA gene
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Although the pathological manifestation may be different, *S. pneumoniae* shares over 99% of 16S rDNA homology with the closely related species, namely *S. mitis* and *S. oralis* . Therefore, classification of these organisms has long been considered difficult. Suppression subtractive hybridisation was performed to search for genomic differences between *S. pneumoniae* and the most closely related species *S. mitis* . About 240 *S. pneumoniae* -specific clonal libraries were evaluated with Southern hybridisation and completely sequenced. Subsequently, *S. pneumoniae* -specific primers were designed and specificities were examined by gradient PCR using genomic DNAs extracted from 132 oral streptococci and other closely related species. Of those primer sets, primers based on cpsA gene amplified only the genomic DNAs from S. pneumoniae strains. Real-time PCR assays designed for the detection of specific sequence regions of cpsA genes were developed (cpsA-rt). The linear regression of standard curves for quantitative real-time PCR assay indicated highly correlations between the log numbers of *S. pneumoniae* cells and the CT values (R2 = 0.99). The limit of detection was 3 pg purified genomic DNA, equivalent to 1000 cells per PCR mixture of a pure culture. These new oligonucleotide primer set may be very useful for the rapid identification and diagnosis to discriminate from other Viridans group streptococci.

P109

Augmented Particle Trapping and Attenuated Inflammation in the Liver by Protective Vaccination against Plasmodium chabaudi Malaria
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Efforts to develop a vaccine against malaria have not been successful yet, reflecting our fragmentary knowledge about protective mechanisms. We have analyzed changes in hepatic gene expression comparing BALB/c mice succumbing to infection with *Plasmodium chabaudi* to those surviving after vaccination. Mice were vaccinated with host cell plasma membranes isolated from *P. chabaudi* -infected erythrocytes. Hepatic and splenic capacity to trap particulate material was determined after injection of fluorescent polystyrol beads. Gene expression in the liver was measured using real-time RT-PCR. In our vaccination model, survival of BALB/c mice was raised from 0% to 80% and peak parasitemia was decreased by about 30%. Vaccination boosted particle trapping capacity of the liver during crisis when splenic trapping is minimal due to spleen ‘closing’. Real-time RT-PCR revelaed that vaccination attenuated malaria-induced inflammation

in terms of expression of the proinflammatory cytokines IL-1, IL-6, TNFα and the macrophage activation markers iNOS and arginase. In contrast, vaccination strongly increased hepatic production of IFN-γ which has been associated with protective TH1 immune responses against P. chabaudi malaria. Moreover, an improvement of liver metabolism, which is severely disturbed during malaria infections, could be detected by measuring expression of the phase II enzyme Sult2a. In particular, vaccination restored inducibility of Sult2a by TCPOBOP, an activator of the constitutive androstane receptor. Our data support the view that the liver is an essential effector site for a vaccine against blood stage malaria: vaccination attenuates malaria-induced inflammation thus improving hepatic metabolic activity.

P110

Investigation of techniques and applications for rapid detection of α-globin gene mutation and deletion

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Aim: Populations in Southeast Asia and South China have high frequencies of α-thalassemia caused by the α-globin gene mutations or/and deletions. This study was designed to find an efficient and simple diagnostic test for α2-globin gene mutations. Methods : A duplex polymerase chain reaction (PCR) assay was designed using two primer pairs: one to amplify the third exon and flanking sequences of the α2 gene and the other to amplify a 78-bp fragment representing the Southeast Asian (SEA) deletion. Denaturing high pressure liquid chromatography (DHPLC) was used to analyze the duplex PCR products at 63.8°C. The study group included 110 samples (80 α-thalassemia samples with various genotypes and 30 normal DNA samples). Results : The PCR products of the sample with known CS/αα, QS/αα and WS/αα DNA showed significantly different profiles, suggesting that DHPLC analysis can directly detect potential mutations. We also detected the SEA deletion, αα or αTα alleles, and -α3.7 and -α4.2 deletions. These results were in accordance with those from multiplex PCR, real-time PCR/dissociation curve analysis, reverse dot-blot (RDB) analysis and DNA sequencing. Conclusion **:** A duplex PCR assay followed by DHPLC analysis can fully diagnose α-thalassemia. This methodology is simple, rapid and accurate, semi-automatic and high-throughput and is thus suitable for large-scale screening.

P111

Two novel approaches used to detect single point mutation causing resistance

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We have developed rapid assays for detection of H274Y in both H1N1 and H5N1 viruses using two novel probe technologies. For the detection of H274Y in H1N1 we have used HydrolEasy® probes. These probes have an increased signal-to-noise ratio and can be designed to have a specific Tm and thereby be designed to have a better sensitivity and specificity than equivalent TaqMan® assays. We have also introduced a novel end-point detection of H274Y in H5N1 based on the novel EasyBeacon™ technology. The end-point detection makes it possible to use standard PCR seetings and equipment (non Real-Time) and make a fast (less than ten minuttes) end-point reading on a Real-Time instrument. In case of a pandemic outbreak is it possible to use this end-point assay for high througput screnning. In contrary to Molecular Beacons, EasyBeacons™ are easy to design. The detection of H274Y alters the recommended treatment and it is therefore important to test for the presence of this mutation following a positive H1N1 or H5N1.

P112

EXPRESSION OF A2A AND A2B ADENOSINE RECEPTORS IN HUMAN BREAST TUMORS

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Background: After identification of the expression profile, signal transduction, molecular function and cell growth modulation of adenosine receptor subtypes in the human breast cancer cell lines, we decided to investigate the possible roles of adenosine receptors in the human breast tissues. In this study, we used RT-PCR to assess A2A and A2B gene expression in normal and tumor breast tissues. Methods: Breast tumors and non-neoplastic mammary tissues (n = 15) were collected immediately after mastectomy and stored at -80°C until use. All tumors were histologically confirmed to be breast cancer. Total RNA was extracted and reverse transcribed to cDNA. PCR primers were synthesized from human adenosine receptor cDNA sequences. PCR was performed under optimized condition for each receptor subtype. Amplification of beta-actin mRNA served as control for RT-PCR. The PCR products were separated on 1.5% agarose gels. Results: To elucidate the expression of A2A and A2B mRNA in breast carcinoma and normal tissues, we compared the level of A2A and A2B mRNA expression by RT-PCR analysis. All breast tumor tissue specimens (n = 11) expressed A2A and A2B adenosine receptor transcripts. In contrast, only one of the four breast specimens from patients without carcinoma expressed no A2A mRNA. Moreover, there were no observable differences between normal and tumor tissues, when normalized against that of beta-actin. Conclusion: In conclusion, these results indicate for the first time, to our knowledge, the expression profile of A2A and A2B adenosine receptors in the human breast carcinoma. However, further studies based on the Real-time quantitative RT-PCR are needed to identify gene expression levels.

P113

Detection of allergens in spiked pasta by Real-time PCR

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Food allergies nowadays represent an important health problem. Undeclared allergens as contaminants in food products pose a great risk for sensitized persons. To ensure compliance with food labelling and protection of consumers reliable detection and quantification methods for food allergens are required. Yet, the detection of allergens in food products can be very challenging, due to the fact that they are often present only in trace amounts or are masked by the food matrix. DNA-based methods are increasingly used for the detection of foreign food constituents. The methods are specific and provide sensitive tools for the detection of specific allergenic components in food. Since reference materials (RM) are scarcely available for food allergens so far, we took an approach to detect five allergens (soy, mustard, celery, lupine, sesame) in spiked pasta using single-plex Real-time PCR. Blank pasta was made from durum wheat semolina and tested for the presence of the five selected allergens. Then spiked pasta was prepared with a starting concentration of 200 ppm for each allergen respectively. Afterwards dilutions were made using spiked pasta material and blank pasta material resulting in concentrations of allergens in spiked pasta material of 200, 50, 20, 10, 5, 1 ppm respectively. DNA extraction was performed using either CTAB or a commercial DNA extraction kit. Real-time PCR was performed on Applied Biosystems 7700 or Corbett Rotorgene using commercial test kits or published methods. Correlation between Ct value and spiked amount of allergen was analyzed for each selected allergen. We showed that correlations are excellent considering the fact that DNA had to be extracted from a food matrix. Limit of detection is about 10 ppm for the selected allergens in this food matrix. Currently we´re analyzing spiked spices as another and more difficult food matrix.

P114

Alternative splicing variants of carbonic anhydese IX in human non-small cell lung cancer

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In human cancer, carbonic anhydrase IX (CAIX) is involved in regulation of intracellular and extracellular pH under hypoxic conditions, and also influences regulation of cell proliferation and tumor progression. CAIX was previously indicated as an independent prognostic marker in non-small cell lung carcinoma (NSCLC). Recently a CAIX alternative splicing isoform was detected in cancer cells independently from hypoxia level. This alternative splicing variant (AS) derived from a transcript lacking exon 8-9, generating a truncated protein lacking the transmembrane region, the intracellular tail and the C-terminal of the catalytic domain, that competes with the full-length isoform (FL) in regulation of the extracellular pH, mainly in a mild hypoxic status. In the present study we measured the mRNA expression of FL, and AS CAIX isoforms in 101 NSCLC and paired not affected tissues. We observed that the two isoforms were coexpressed in all NSCLC and normal tissues, but AS mRNA was prevalent in normal tissues (66±3%), while the FL isoform was higher in NSCLC (58±2%, p=0.001). FL mRNA, and not AS, was statistically increased in NSCLC (p=0.01) and showed a statistical association with lymphnode involvement (p=0.009) and tumor stage (p=0.04). Global analysis of cancer/related death showed that high levels of FL mRNA were predictive of unfavourable outcome (p<0.0001) and shorter desease-free survival (p<0.0001). In NSCLC, multivariate analysis indicated that FL is an independent prognostic factor for overall survival, and higher levels of FL mRNA sensibly increase hazard ratio (approximately six fold). In conclusion, our results seems to indicate that FL CAIX is the most accurate surrogate of hypoxic stress and represents the only variant with a prognostic role in NSCLC. These data indicate the importance of a separate measurement of the two isoforms in cancer and the need of an accurate re-evaluation of the most studies on the clinical role of CAIX in cancer diagnosis.

P115

High- resolution melting analysis for rapid detection of KRAS, BRAF, and PIK3CA gene mutations in colorectal cancer

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High Resolution Melting Analysis (HRMA) provides a new valid approach to efficiently detect DNA genetic and somatic mutations. In this study HRMA was used for the screening of 116 colorectal cancers (CRCs) to detect hot-spot mutations on the PIK3CA gene, KRAS and BRAF oncogenes. In particular for PIK3CA, mutational hot spots on exon 9 and 20 were analysed, for BRAF on exon 15 and for KRAS on exon 1. Direct sequencing was used to confirm and characterized HRMA results. HRMA revealed abnormal melting profiles in 65 CRCs (56.0%), 16 of them harbouring mutation in two different genes, simultaneously. The frequency of mutations was 17.2% for PIK3CA (11.2% in exon 9 and 6.0% in exon 20), 43.1% for KRAS exon 1, and 9.5% in exon 15 of the BRAF gene. We found a significant association between PIK3CA and KRAS mutations (p=0.008), whereas KRAS and BRAF mutations were mutually exclusive (p=0.001). This report describes a novel approach for the detection of PIK3CA somatic mutation by HRMA.

P116

Multi-diagnostic qPCR assay for rapid detection of the most frequent human pathogens associated with Sepsis.

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Sepsis is a syndrome commonly called "blood stream infection" defined by the presence of bacteria (bacteremia) or other infectious organisms and/or their toxins in the blood (septicemia). Sepsis is commonly associated with clinical symptoms of systemic illness characterized by a generalized inflammatory response due to the above mentioned bloodstream infection, and is one of leading killers in general intensive care unit population. For the treatment of septic patients, it is important to perform a rapid and accurate identification of the causative microorganisms of the syndrome, in order to use the appropriate medicine. Growth in liquid media is the conventional method for detecting microorganisms associated with this syndrome. However, the blood culture method requires several days to detect and identify the bacteria and to run a later test for susceptibility to antibiotics. Therefore, in the absence of an appropriate diagnostic test, the sepsis-treatment involves broad-spectrum antibiotics in advance of the test, which are seriously increasing resistance to antibiotics as well as the costs related to its treatment.
In the last years, the development of qPCR has provided a fast and reliable method for detecting the presence of specific taxa in different environments. The aim of this project was to design and develop a new sensitive method for the diagnostic of sepsis by qPCR assay from potentially positive blood cultures. In order to achieve this goal, we have used LionProbes® , owned by Biotools B&M Labs, S.A., to detect amplified PCR products belonging to specific bacterial pathogens. Polymorphisms in 16S rRNA gene have been selected to design genus-specific probes for the detection of *Escherichia coli* , *Klebsiella pneumoniae* , *Staphylococcus aureus* and *Streptococcus pneumoniae* , the main microorganisms associated with sepsis under common hospital conditions.
We have adapted the qPCR assay to a gelified 96-well plate format to increase the throughput and the use of the gelification technology in order to stabilize the reaction mixture. Gelification® technology developed by BIOTOOLS B&M Labs, S.A., is a stabilization procedure for reagents and reaction mixtures mediated by the addition of a stabilizing mixture and the partial removal of water present in the gelification solution. This stabilization method shows advantages for storage, transportation and minimal manipulations, as well decreasing the risk of contaminations and pipetting errors.
Here we present the development and application of a multi-diagnostic qPCR assay that is less time consuming and cost-effective, providing rapid results that allow the application of a fast and effective medical treatment, reducing the patient's exposure to unnecessary or ineffective antibiotics.

P117

A Quadraplexed Real-Time PCR Assay for Rapid Detection and Differentiation of the Clostridium botulinum toxin genes A, B, E, and F in Pure Culture and Environmental Samples

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Clostridium botulinum is the etiologic agent of botulism, a disease marked by flaccid paralysis that can progress to asphyxiation and death. This species of bacteria produces the most potent toxins known with an LD50 in primates of 1-10 ng kg-1 of body weight. Because of their potency, these toxins have the potential to be used as biological weapons. Therefore, C. botulinum has been classified as a select agent by the United States Centers for Disease Control and is considered to be equally dangerous by other governments. There are four related but antigenically distinct botulinum toxins that cause disease in humans (A, B, E, and F) and these can enter the body via three different routes: inhalation, ingestion, and absorption from wound infections. Ingestion of C. botulinum spores by infants has been associated with sudden infant death syndrome. The mouse bioassay is the current gold standard by which toxin type is confirmed. However, this method is expensive, slow, and very labor intensive, taking up to four days to complete. In addition, this assay carries ethical concerns due to the need to sacrifice mice. Commercial biochemical tests have failed in identifying various toxin-producing strains of C. botulinum. PCR-based assays have been used extensively for the detection of botulinum toxin-producing bacteria in food, animals, and fecal samples, and recently, to help diagnose disease in humans. Most of these are traditional PCR methods, though assays have been published in recent years that use real-time PCR to target one or

more botulinum toxin genes. However, no assay has been published that involves real-time PCR detection of the four human disease-causing toxin genes A, B, E, and F in a single-tube, multiplex reaction. This report describes the development of a real-time PCR single-tube assay that uniquely identifies these four botulinum toxin types responsible for human disease. A total of 83 C. botulinum isolates were evaluated in this study, as well as numerous near-neighbors and other bacterial species. Included were isolates which had genes for each of the toxins A, B, E, and F with some natural isolates containing genes for more than one toxin. C. botulinum isolates producing the toxins C and D, which do not cause disease in humans, were also included as controls. Results showed that this quadraplexed assay was capable of detecting any of the four toxin genes in a given sample at a sensitivity of about 100-200 pg of genomic DNA. Furthermore, it was able to detect the presence of two, three, or all four toxin genes in a given sample, indicating the lack of type-to-type interference. The test was also functional in the presence of extraneous organic matter commonly found in various environmental samples. This assay could prove to be a useful tool in the rapid identification of a specific type of disease, or the potential toxic threat of a substance to human health.

P118

Alteration of the CEACAM1-Splice Variant Expression in Bladder Carcinoma

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Objective: Diagnostic and prognostic biomarkers in bladder carcinoma, which assess early non-invasive tumours in their clinical course, help tremendously to decide for the optimal treatment of the patient. Carcinoembryonic Antigen-related Cell Adhesion Molecule 1 (CEACAM1) is known to be down-regulated in epithelial cells of various tumours and acts as a proangiogenetic factor which could be involved in the tumour transition from a non-invasive to an invasive phenotype. The aim of the present study was to characterize the four major splice variants of CEACAM1 in the progression of bladder cancer and to analyze their prognostic potential as tumour markers.
Patients and Methods: 95 cases of bladder carcinoma patients (30 pTa, 15 pT1, 11 pT2, 29 pT3, 10 pT4) and four non-cancer patients were chosen. For each CEACAM1 splice variant a specific quantitative RT-PCR was designed and performed. The expression data were analyzed using Mann-Whitney-rank sum.
Results: It could be demonstrated for the first time, that CEACAM1-3L is the predominant splice variant in normal tissue. In comparison, all samples of bladder cancer tissue consisted of significantly less CEACAM1-3L ($p<0,001$) and CEACAM1-3S ($p=0,006$). Additionally, CEACAM1-4S ($p=0,005$) and CEACAM1-3S ($p=0,013$) expression was significantly higher in pT1 than pTa. The overall amount of CEACAM1-S and CEACAM1-L in normal tissue was the same (ratio S/ L=1,67), while in all bladder carcinoma there was significantly more S (ratio S/L >7).
Conclusion: These data suggest, that the expression of CEACAM1 splice variants change during transition from normal transitional epithelium to tumour and probably also from non-invasive to invasive and vascularised tumour type. With these properties, the determination of CEACAM1 splice variants seems to be a potential marker for bladder carcinoma. Since CEACAM1 is involved in various other tumours, such as colon carcinoma, leukaemia or prostate cancer, it will be valuable to expand the characterization of the CEACAM1 splice variants to other normal and pathological tissues.

P119

Use of real-time quantitative RT-PCR (qRT-PCR) for assessment of chemical effects on receptivity of human endometrium for embryo implantation

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The identification of reproductive toxicants and their mechanisms of action is a major scientific challenge during safety assessment of chemicals and the process of drug prioritization. Our work is part of the integrated EU-project ReProTect developing new *in vitro* tests required under the new European chemicals legislation (REACH). We investigate effects of chemicals interfering with the receptivity of human endometrium for embryo implantation. As highly sensitive method qRT-PCR is applied to detect chemical effects on mRNA level. As models the human endometrial Ishikawa cell line and human endometrial explants cultures are investigated.
In order to identify predictive toxicological endpoints among a broad spectrum of possible target genes, assays from the Universal Probe Library (Roche) were used on a LightCycler 480 instrument. Data analysis was performed by calibrator-normalized relative quantification with efficiency correction. Reference genes were selected by a systematic, software-based approach (NormFinder, geNORM). Standard curves for low and medium expressed genes were established by addition of standard DNA generated by conventional PCR to the sample matrix.
The successful operation of the calibrator-normalized relative quantification method was demonstrated by studying mRNA levels of progesterone (PR) and estrogen receptors (ER), leukaemia inhibitory factor (LIF) and cyclooxygenase-2 (COX-2) before and after endometrial explants culture. mRNA levels of PR and ERα were reduced and those of LIF and COX-2 increased after 6 hrs of culture. Quantitative dose-response-relationships were established for the up-regulation of progesterone receptor mRNA by various estrogenic compounds in the human endometrial Ishikawa cell line. Specific time courses were elaborated for the expression of selected target genes under the influence of various test substances. Whereas for progesterone and estrogen receptors a continuous increase of mRNA levels was found, for LIF a peak mRNA expression after 1-2 hours after addition of progestagenic test compounds was observed.
In summary, qPCR has been demonstrated to be a sensitive and rapid method for describing dose-response-relations and time courses of effects of environmental compounds on mRNA levels of pre-selected target genes.
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P120

PROSTATE CANCER SPECIFIC mRNA/miRNA DETECTION IN CELL-FREE PATIENT URINE BY REAL TIME RT-qPCR

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Objectives : Human patient urine contains a cell-bound and a cell-free RNA (ufRNA) component. Proliferating urogenital tumors (kidney, bladder, prostate) shed RNA molecules (mRNA, miRNA) into urine by various physiological mechanisms: apoptosis, necrosis and cell lysis. We postulate that the prostate tumor specific ufRNA fraction could mimic the aberrant cancer RNA expression signature of the malignant tissue. To investigate the hypothesis we surveyed selected biomarker RNAs in the cell free urine supernantant of cancer patients and a non-diseased control group using SYBR Green I real time qPCR together with validated reference genes.
Methods : Cell-free urine was prepared from consenting young males (aged below 35 years of age) and patients undergoing routine surgery for benign prostatic hyperplasia (BPH) and prostate cancer by low speed centrifugation. Total RNA extraction was performed with silica filter based protocols (RNeasy Mini kit, Qiagen; ZR Viral RNA Extraction Kit, ZymoResearch) and by a phenol/chloroform extraction method (peqGold RNA Pure FL). RNA content was quantified with a Quant-iT RNA assay kit using a Qubit fluorometer (Invitrogen). cDNAs were prepared with a Transcriptor First Strand cDNA kit (Roche Diagnostics) or a miRNeasy Mini Kit (Qiagen) and real time PCR experiments were performed on a LightCycler® 480 instrument using SYBR Green I for amplicon detection. The three best reference genes validated by a statistical geNORM procedure (GenEx Professional Software,

MultiD) were used for normalizations (geNORM house-keeping gene selection kit, PrimerDesign).
Results **:** A geNORM analysis of 9 reference genes with four different urine types (normal urine collected from young males aged below 35 years, post-DRE BPH, pre-DRE and post-DRE cancer) revealed the overall best fitted gene pair for relative quantifications (RPLPO, GAPDH; M = 0.696). From the investigated markers the qualitatively most abundant RNAs were PSA, RPS2 and hepsin: normal (PSA: 2/9; RPS2: 7/9; hepsin: 1/9), pre-DRE BPH (PSA: 4/5; RPS2: 5/5; hepsin: 3/5), post-DRE BPH (PSA: 8/9; RPS2: 9/9; hepsin: 4/9), pre-DRE cancer (PSA: 5/16; RPS2: 15/16; hepsin:4/16) and post-DRE cancer urine (PSA: 14/16; RPS2: 16/16; hepsin: 7/16). A qPCR real time statistical analysis of the expression of PSA, RPS2, five novel patented marker genes (A to E) and six selected miRNAs (miR-202_2, miR-210_1, miR-320_3, miR-373*_1, miR-498_1, miR-503_1) did not significantly differentiate BPH from cancer urine in a small patient group.
Conclusions : Urine cell-free RNA is a significant diagnostic resource for prostate cancer biomarker analysis. We report two novel findings: prostate-derived ufRNA is enriched in post-DRE patient urine and the miRNA component represents a potential novel resource for a diagnostic assay development. (supported by OENB P11491).

P121

Additional step in detection of free fetal DNA from maternal plasma to improve non-invasive prenatal diagnostic

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Rhesus D (RhD) blood group incompatibility between RhD-negative mother and RhD-positive fetus can occasionally result in maternal alloimmunization where the resultant anti-D can cross the placenta and attack the fetal red cells, which in worse case scenarios can cause fetal anemia and ultimately death. Modern non-invasive prenatal diagnostic have been developed using free fetal DNA circulating in the maternal blood of pregnant women to determine fetal *RHD* genotype. The isolation and detection of free-fetal DNA is critical because of low yield of isolated free fetal-DNA. In our study for isolation of free-DNA the automatic isolation by EZ1 instrument (Qiagen) using QIAamp Virus kit (Qiagen) was used. A new preanalytic step was included for improvement in test robustness for reliable results in early pregnancy. The isolated free-DNA was preamplified with PreAmp Master Mix (Applied Biosystems). The real-time PCR TaqMan assays for exon 7 of *RHD* gene and/or *SRY* gene and *albumin* gene were used for non-preamplified DNA and preamplified DNA. In our study the comparison between the different approaches was made.

P122

Expression of IBSP, COL15A1 and WISP-1 in osteoporotic bone tissue

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Studies, based on the genetics of osteoporosis, play an important role in clinical practice. Osteoporosis, a major public health problem, is a systemic disease of the skeleton, characterized by low bone mineral density (BMD) and alterations in bone microarchitecture with a low-energy fracture in the area of hip, wrist and spine as a main clinical end-point. Testing for genetic markers in early youth could help determine the risk of developing osteoporotic fractures in later life. Genes that regulate BMD and bone fragility are potential targets for the synthesis of new drugs, which could be used for the treatment of osteoporosis. Furthermore, they could be used also as new diagnostic and prognostic markers for osteoporosis.
A preliminary study using DNA microarrays, discovered several genes, which were expressed differenty in osteoblasts from osteoporotic as opposed to osteoblasts from non-osteoporotic bone tissue. The purpose of our work was to measure the expression of three differently expressed genes (IBSP, COL15A1 and WISP-1) in the bone tissue, attempting to confirm the involvement of the genes in the pathogenesis of osteoporosis.
71 patients were involved in the study. Spongious bone tissue was sampled in the proximal femur. BMD was measured at the total hip, femoral neck and lumbal spine, using dual-energy x-ray absorptiometry. qPCR was used to measure the expression of genes of interest. PPIA and RPLP were experimentaly choosen as reference genes. SYBR Green was used for product detection, with the exception of WISP-1, where TaqMan probes were employed. Using standard curves for each gene, we determined the relative concentrations of mRNA of each gene in the samples. We then normalized the expression of each gene from each individual sample with the reference genes. Finally, we statistically evaluated the clinical significance of IBSP, COL15A1 and WISP-1 gene expression using non-parametric statistics.
IBSP was 3.2-fold up-regulated in osteoporotic tissue (p=0.036) when BMD of the total hip was considered. When the BMD of the femoral neck and lumbal spine were considered, the up-regulation was 2.4-fold (p=0.096) and 1.3-fold (p=0.802), respectively. WISP-1 was up-regulated only when hip BMDs were considered (4.6 – 3.2-fold up-regulation), however it did not reach statistical significance. No difference was observed in the expression of COL15A1.
Our study represents a further step in researching the pathogenesis of osteoporosis, as well as a contribution to the development of new solutions for diagnosing and treating the illnesses of bone tissue.

P123

RAPID DETECTION OF HUMAN INFLUENZA VIRUSES IN ONE STEP RT-qPCR PORTABLE MICRODEVICE

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In this work we have developed a portable microfluidic device for a specific, rapid and early detection of human influenza viruses in clinical samples (nasopharyngeal and throat swabs). The microdevice is able to generate cDNA and qPCR amplification of influenza molecular markers in one step RT-qPCR taking into account the lab on a chip concept. The biochemical reaction is carried out in a l SU-8 microchamber, up to 40 minutes.μsingle 3
Influenza viruses adapted to human have caused significant pandemic or epidemic waves. In addition, these viruses cause several thousands of deaths each year in Europe. An emerging outbreak is produced by avian influenza virus that can also be transmitted to humans. This is the cause of great economic losses, million of euros in Europe. Therefore there is a real need for a test providing fast identification of the influenza causative agent and thus contributing to the improvement of treatment and surveillance. Nucleic acid analyses are the most appropriate assay scheme for both early detection and late surveillance of influenza. RT-qPCR on influenza RNA samples is a fast and sensitive method to detect the presence of influenza genetic material in clinical samples. For this work primers based on matrix gene (M gene) have been designed for the universal detection of target viruses and subtypes as A and B influenza.
The microfluidic device consists of a chip (1), with a single microchamber of 3 mm wide and 5.4 mm long for a 3 µl RT-qPCR reaction volume. RT-qPCR mastermix containing RNA isolated from clinical samples is pipetted into the chamber, a chip with electrodes. The chip is then introduced into the capsule that is connected to a small device able to generate the thermocycling steps. The emitted fluorescence signal is captured in real time by a photomultiplier tube through the microchip cover and fluorescence signal is analysed with a data acquisition unit. The one step RT-real time PCR method shows a high specificity and accuracy. The end point DNA concentration after RT-qPCR of assays carried out on chip was very similar to experiments performed on conventional thermocycler. These preliminary assays have been done using universal primers for the amplification of the matrix gene of IVA. Next assays will include primers for several sub-types and an internal amplification control. This work proves our developed portable microdevice is able to carry out the influenza detection by RT-qPCR. In a next future we will include a RNA isolation step from clinical samples, in order to provide a more integrated lab on a chip microdevice.
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(1) Aguirregabiria et al., Proceedings of uTAS 07, 584-586 (2007)

P124

Expression levels of type III and VI mRNA transcripts from TMPRSS2-ERG gene fusion in cancerous and benign prostate tissue by qRT-PCR

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OBJECTIVES - Novel prostate cancer markers are needed for improved diagnosis and prognosis of this heterogeneous disease. Transcripts from a gene fusion between *TMPRSS2* and *ERG* have lately been discovered in subgroups of prostate cancers. Our aim was to study the clinical applicability of the most frequently expressed transcript (type III) and the transcript suggested to be associated with aggressive prostate cancer (type VI) by determining their expression levels in benign and malignant human prostate tissue samples obtained immediately after radical prostatectomy. The expression levels were also compared to *KLK3* mRNA counts.
METHODOLOGY - Quantitative real-time reverse-transcription PCR (qRT-PCR) assays were developed for *TMPRSS2-ERG* transcripts types III and VI. Assays were based on a generic closed-tube protocol developed at our department. The real-time detection in PCR is based on time-resolved fluorometry by utilizing lanthanide chelate-labeled target specific probes and separate quencher probes. In addition to using external standard curves, artificial RNA molecules are added to samples to serve as internal standards. A sample panel consisting of GITC-stabilized radical prostatectomy tissue samples from 88 prostate cancer patients was analysed. Two samples were collected from each prostate aiming at the cancerous site and a benign control sample. Microscopic examination of the adjacent tissues was performed to confirm the pathology of the samples. Also, expression level of *KLK3* mRNA was determined with a similarly constructed assay from the same samples.
RESULTS - Detection limits for the *TMPRSS2-ERG* type III, *TMPRSS2-ERG* type VI and *KLK3* mRNA assays were 5, 20 and 2.5 mRNA copies per µl of cDNA template, respectively. Using binary classifications, fusion transcript type III was found in 42 % of the benign samples and in 56 % of the cancerous samples (p=0.06) and type VI was found in 30 % in the benign group and in 51 % of the cancerous samples (p=0.007). The fusion transcript concentrations per µg RNA of the positive cancerous and benign samples did not differ significantly from each other (type III, p=0.10 and type VI, p=0.45). *KLK3* mRNA expression was not found to be changed from benign to malignant tissue.
CONCLUSIONS - This is a first report of a truly quantitative assay for the determination of *TMPRSS2-ERG* type III and type VI fusion transcripts in cancerous and benign tissue samples obtained early after radical prostatectomy. The frequency of prostate cancer samples positive for the *TMPRSS2-ERG* fusion transcripts is similar to previously reported results. The high positivity and expression levels of the fusion transcripts in the benign tissue samples was unexpected, and may be explained by lack of true non-cancerous homogeneity and/or a field effect from the cancer site. Further studies with samples from non-cancer patients are needed to resolve this question.

P125

Introducing a new biomarker for schizophrenia

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Schizophrenia, commonly developed in adolescents and young adults, is one of most common mental disorder, but the phatophysiology and etiology of schizophrenia is still obscure. Numerous studies on dopamine and schizophrenia have suggested that change in the dopamine system is related to schizophrenia, but there is little direct evidence for dopamine hypothesis in schizophrenia.
Recent progress in molecular biology, and imaging technique has enable new insight for schizophrenia research. Changes in the dopamine system are influence not only by dopamine itself, but also by dopamine receptors.
Recent progress in molecular biology reveals the existence of mRNA of D3 and D4 dopamine receptor in peripheral lymphocyte. However, the clinical significance of these finding and whether or not these receptor reflect central dopamine receptors remains uncertain.
The purpose of this study were to examine if the mRNA of the peripheral dopamine receptor is changed in schizophrenia patients.
50 Naive patients were enrolled. After extracting RNA from white blood cells, cDNA is synthesized.
After doing the quantitative Real-time PCR, expression of D3 receptors in healthy persons and patients were compared.
Results of this examinations reveals that expression of D3 receptor is increased in compared with controls sample.

P126

Differential Expression of hypothetical genes in a biochemical morphotype of the marine sponge Discodermia dissoluta

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The marine sponge *Discodermia dissoluta* has been shown to produce a variety of secondary metabolites, some with promising antitumor properties such as discodermolide. Suppression subtractive hybridization (SSH) experiments with two *D.dissoluta* biochemical morphotypes have yielded a large expressed sequence tag (EST) dataset of 1,536 transcripts including eukaryotic, prokaryotic, and viral genes. Preliminary analysis of the resulting transcripts indicated a profile of 880 eukaryotic, 325 prokaryotic, 21 viral, and 44 unknown ESTs. Of these, 538 represent hypothetical genes, some of which are the focus of this study.
We have analyzed differential gene expression using quantitative polymerase chain reaction (qPCR) with SYBR Green chemistry to screen and characterize genes, some of which may be related to novel metabolism and unknown functions related to symbiosis within the complex sponge-microbial community. Preliminary data indicate differential expression of selected hypothetical genes in the secondary metabolite positive morphotype. Bioinformatics analysis of these transcripts also provides insight into their potential function and ecological roles within the complex sponge microcosm.
This information will in turn allow researchers to screen for novel drug candidates by probing for specific genes involved in secondary metabolite production. Screening for specific genes requires substantially less material than traditional analytical chemistry methods which require a large amount of biomass. Additionally, elucidation of the genes potentially driving the production of bioactive substances will aid in their synthesis in the laboratory. This will eliminate the need for large scale harvesting of organisms, thereby conserving the natural environment.

P127

Highly sensitive qPCR for the JAK2-V617F mutation to guide adoptive immunotherapy in patients with myelofibrosis who received allogeneic stem cell transplantation

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During the last few years allogeneic stem cell transplantation (allo-SCT) after dose-reduced conditioning has been established as a promising treatment option for myelofibrosis. However, relapses of the malignant disease after transplantation remain a significant problem. Single-case reports on successful donor lymphocyte infusions (DLI) for relapse treatment after allo-SCT have provided evidence for a graft-versus-myelofibrosis effect. In this study we investigated the potential of our recently developed highly sensitive JAK2-V617F-specific real-time PCR to guide adoptive immunotherapy in myelofibrosis patients after allo-SCT. Therefore,

the clinical impact of escalating DLI was compared in 17 patients with myelofibrosis who had either a clinical relapse (salvage-DLI, n=9) or a molecular relapse / molecular residual disease (pre-emptive DLI, n=8) after dose-reduced allo-SCT. Thirty-five DLI from related (n=5) or unrelated donors (n=12) were given with a median dose of 1x106 for the first and 5 x 106 CD3+cells/kg for the second DLI. The complete molecular response rate was 68 %, and significantly higher for patients who received DLI for molecular than for clinical relapse (100 % vs. 44 %) (p=0.04). In comparison to molecular relapse, clinical relapse required more donor-lymphocyte infusions (two vs. one) and was associated with a higher incidence of acute GvHD (22 % vs. 0 %). Notably, two of the four patients who achieved complete molecular remission in the salvage group had developed an acute GvHD (grade III), whereas no GvHD was documented in non-responders. These observations confirm earlier data on a strong donor T cell-mediated graft-versus-myelofibrosis effect. In conclusion, the molecular monitoring of minimal residual disease using a highly sensitive qPCR for the JAK2-V617F-mutation represents an efficient mean to guide adoptive immunotherapy in myelofibrosis patient. This approach increases clinical efficacy of donor-lymphocyte infusions while decreasing their toxicity. We therefore suggest that qPCR-triggered adoptive immunotherapy should be implemented in further clinical trials.

P128

NICOTINAMIDE N-METHYLTRANSFERASE: A POTENTIAL PROGNOSTIC MARKER FOR ORAL SQUAMOUS CELL CARCINOMA.

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Oral squamous cell carcinoma (OSCC) is the most common malignancy of the oral cavity, representing 90 % of all oral cancers. Despite advances in diagnosis, surgical techniques, general patient care, and local and systemic adjuvant therapies, the mortality rate of OSCC has shown little improvement over the last three decades and the overall five-year survival of these patients remains less than 50%. The high mortality from OSCC is attributed to the presence of cervical lymph node metastasis and the diagnostic delay seems to be responsible for the poor prognosis of patients with OSCC. Therefore, there is an imperative need for sensitive biomarkers to improve early detection of this malignancy.
In the present study, we focused on the expression of genes critical in the drug metabolism process, namely on Nicotinamide N-Methyltransferase (NNMT), enzyme belonging to Phase II Metabolizing Enzymes. To explore the involvement of NNMT in OSCC, we analysed the enzyme expression in paired tumour (T) and non-tumour (NT) tissues obtained at surgery by semiquantitative RT-PCR, Real-Time PCR, western blot and immunohistochemical analyses.
Compared with normal mucosa, OSCC exhibited significantly increased expression of NNMT in 11 of 22 (50 %) examined patients. Interestingly, NNMT was upregulated in most of the favourable OSCCs (N 0), while no marked NNMT expression alterations between tumour and normal mucosa were detected in most of the unfavourable OSCCs (N+). Both, pT and pathological staging showed an inverse correlation with NNMT mRNA levels, and a significant negative association of the amount of NNMT expressed by tumour tissue compared to the adjacent normal mucosa was found with metastasis (1).
We also evaluated the effect of shRNA-mediated inhibition of NNMT on the proliferative potential and apoptosis of oral cancer cell line PE/CA-PJ15. The efficiency of gene silencing was detected by Real-Time PCR and western blot analysis.
The cell proliferation inhibition was determined by MTT and soft agar colony formation assays; cell cycle distribution and apoptosis were examined by flow cytometry. ShRNA vectors targeted against NNMT efficiently suppressed gene expression, showing inhibition rates around 70 %, observed at both the mRNA and protein levels. The shRNA-mediated gene silencing of NNMT resulted in a significant rise in apoptosis rate.
The present data support the hypothesis that the enzyme plays a role in tumour expansion, and NNMT expression level measurements would provide a rapid and useful method of identifying patients at high risk of lymph node metastases. Therefore, NNMT may have potential as a new prognostic marker, and its inhibition could represent a possible molecular approach to the treatment of OSCC.

1. Sartini D et al. (2007) MOL MED 13, 415-421.

P129

NEUROTROPHINS AND RECEPTORS EXPRESSION PROFILING OF PLACENTAS FROM PREGNANCIES COMPLICATED BY HELLP SYNDROME AND INTRAUTERINE GROWTH RESTRICTION (IUGR)

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OBJECTIVE: The neurotrophin family comprises molecules involved in growth, differentiation, survival, diseases, regeneration and normal functions of the neuronal system. Particularly, neurotrophins play an important role in pre-natal and post-natal brain development due to their neuro-protective action.
Our aim was to investigate the expression pattern and the role of neurotrophins and their receptors in the placentas from pregnancies complicated by HELLP (Hemolysis, Elevated Liver enzymes and Low Platelet count) syndrome and intrauterine growth restriction (IUGR).
STUDY DESIGN: Placentas from normal term pregnancies (n=15), pregnancies complicated by HELLP syndrome (n=10) and intrauterine growth restriction (IUGR) (n=10) were collected.
Macroarray analyses were performed with GEArray Q Series Human Neurotrophin and Receptors Gene Array HS-018 (SuperArray Bioscience Corporation, Frederick, MD). The data were confirmed by quantitative Real-Time PCR. The Student's test was used for statistical analysis. Differences were considered significant at $p < 0.05$.
RESULTS: The expression of 10 (HELLP) and 6 (IUGR) genes, respectively, was significantly different in the pathological group compared to control. Particularly, 3 genes were up-regulated and 3 down-regulated in the IUGR group while 6 genes were up-regulated and 4 down-regulated in the HELLP group. Differential gene expression measurements (HELLP versus normal, and IUGR versus normal), performed by real-time PCR technique, revealed a significant down-regulation ($P<0.05$) for STAT3 (signal transducer and activator of transcription 3) and its isoforms STAT3α and STAT3β (fold change values between 1,13 and 2,32).
CONCLUSIONS: A variable capacity of producing some neurotrophins could be involved in the pathogenic mechanism leading to pregnancies complicated by HELLP syndrome and IUGR.
Our data seem to suggest that STAT3α could play a key role as common denominator for all important factors involved in successful pregnancy outcome (e.g. "IL-6 cytokine family").
In addition, the observed STAT3β down-regulation underlines the relevance of this isoform in regulating a different subset of target genes, which appear play trophic roles in decidual development.

P130

Direct and Fast DNA Methylation Quantitation

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DNA methylation is the most important epigenetic alteration involved in normal cellular processes as well as diseases. In cancer research, aberrant DNA methyaltion has been discovered as marker for cancer diagnostics and prognosis.
Detection and quantitation of methylated DNA often starts with bisulfite conversion of non-methylated cytosines to uridines, leaving methylated cytosines unchanged. The differences between methylated and non-methylated sequences can be then analyzed by various assay formats.
We have developed a universal bisulfite conversion protocols to convert cytosines in DNA that are either purified or in cells. The whole process, including cell lysis, bisulfite reaction, desulfination, and purification, can be completed in two hours. In combination of high resolution melting analysis, down to 1% methylated DNA can be quantified directly from cell mixtures.

P131

Application of qPCR Assays to the Investigation of New Classes of Antiretroviral Drugs in the Treatment of HIV/AIDS

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Background: Highly active antiretroviral therapy (HAART) has been very successful in the treatment of HIV-1 infection. However, due to rapidly emerging drug-resistant virus, new classes of drugs are continually required, ideally targeting a different stage of the retroviral lifecycle. Drugs that target the HIV-1 integrase, a critical enzyme for viral replication, have recently been approved for use. Due to their unique action against this stage of viral replication, clinical trials with HIV-1 integrase inhibitors have shown excellent results. Paradoxically, by preventing integration, integrase inhibitors result in a build-up of unintegrated DNA that is transcriptionally active and can produce early HIV-1 gene products, with unknown consequences. Methods and Results: We have developed Taqman probe-based qPCR assays to quantify different DNA forms present throughout the retroviral lifecycle, including early and late reverse transcription products, unintegrated DNA circles, and integrated DNA. We have also developed a qRT-PCR assay to detect all of the ~23 mRNA splice variants of early HIV-1 gene products, which can be produced from unintegrated DNA. These assays have been applied to cell culture experiments involving treatment of CD4+ T cells or macrophages with inhibitors of HIV-1 reverse transcriptase or integrase. Results will be presented that show the impact of inhibiting different stages of viral replication, in an aim to dissect the enhanced clinical efficacy shown by HIV-1 integrase inhibitors. Additionally, results will be presented showing the effect that inhibiting integration with clinically approved antiretroviral drugs has on the production of early viral gene products from unintegrated DNA, to complement other studies by our group into their potential effects on both infected and uninfected cells. Discussion: By applying q(RT)-PCR assays to cell culture experiments that include the treatment of HIV-1 with different classes of antiretroviral drugs, we hope to further understand the extremely promising results shown by integrase inhibitors for the treatment of HIV/AIDS. This will be useful in the development of future antiretroviral drugs, and hopes to shed light on strategies aimed at limiting the establishment of the latent reservoir of HIV-1, which represents the ultimate barrier to eradication of HIV-1 infection.

P132

Development of a RQ-PCR Assay for quantitative detection of viruses like Simian Virus 40, as well as for dental pathogens like Streptococcus mutans

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AIM: Simian virus 40 (SV40)is known to posess strong oncogenic potential, based on the large T-antigen (TAG). Previous studies detected SV40 in a variety of human malignancies, so in lymphomas, and osteosarcomas, but with significant discrepancies in its frequency. Differences in DNA quality and in virus detection methods hamper a controlled and standardised analysis. We establish a RQ-PCR based TaqMan assay for rapid and highly reproducible detection and quantification of SV40 and used it for analysing samples from childhood malignancies. Streptococcus mutans (S. mutans) has been consistently linked with the formation of dental caries, one of the most common infectious diseases afflicting humans. Moreover it is associated with non oral infections, so to endocarditis. Various methods have been developed to identify oral bacteria, but rare quantitative analysis are done up to now. In spite of the importance of S. mutans as cariogenic dental pathogen our aim was to establish a RQ-PCR based TaqMan assay for detection and quantification of S. mutans in oral specimens. METHODS: DNA was extracted either from fresh cells, or dental plaque, using the Quiagen spin column method. RQ-PCR primer were selected in case of SV40 for the retinoblastoma pocket binding domain of TAG and in case of S. mutans for the glucosyltransferase B (gtfB)-gene. The internal TaqMan probes were each labelled with FAM at the 5`end and TAMRA at the 3`end. RQ-PCR was performed in 10µl reactions with 500ng template DNA, 1 U Taq-Polymerase, and annealing temp. of 60oC (SV40) or 58oC (S. mutans. DNA was serial diluted (10-1 to 10-6) and samples tested double or triple. Positive samples were analysed additionally on agarose gels, and in part sequenced by using the Abi Prism BigDye terminator cycle sequencing kit. RESULTS: For SV40 we analysed tumour-, blood- and bone marrow- samples from a variety of malignancies as well as blood from healthy donors with this real-time quantitative assay. Overall we found SV40 virus in 22% osteosarcomas, 76% lymphomas, 21% Breast cancer and 1,4% healthy individuals from German origin. In samples from Hungarian origin we found in 74% osteosarcomas and 18% healthy individuals SV40 positivity. For S. mutans we analysed plaque samples with this RQ-PCR assay from a variety of donors, those who consumed chewing gums with sugar or with Xylit as a sugar substitute, and compared both groups with those never using chewing gum, in order to get quantitative differences in S.mutans bacteria. CONCLUSIONS: Here we present a RQ-PCR based assay for standardised detection and quantification of virus as well as bacteria sequences which can also be used for detection and quantification of other viruses or bacteria. The use of 500ng template DNA in each reaction and the exact quantification allows the sensitive detection of virus sequences down to 4 log as well as for bacteria down to 6 log. Financially supported by Kinderkrebszentrum Hamburg e.V.

P133

Differential gene expression analysis of APP isoforms in platelets from patients with Alzheimer's Disease and healthy controls.

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Alzheimer disease (AD) is a chronic neurodegenerative disorder characterized by a progressive cognitive and memory decline. In the past few years, different biochemical parameters in cerebral spinal fluid (CSF) or plasma have been investigated. It has been tried to identify peripheral markers of AD, focusing mostly on the amyloid precursor protein (APP). Different isoforms of APP are generated by alternative splicing. The three major isoforms are constituted of 770, 751 and 695 aa residues. APP 751 and APP 770 contain a Kunitz-type serine protease inhibitor domain (APP-KPI), and APP 695 lacks this domain. In this regard, platelets represent an important peripheral source of APP. It has been demonstrated that three major APP isoforms with apparent molecular weight ranging from 106 to 130 kDa are inserted in the membrane of resting platelets and that both platelets and megakaryocytes express three transcripts encoding for the isoforms: APP 770, APP 751, APP 695. Several studies independently described alterations in APP metabolism/concentration in platelets of Alzheimer's Disease patients when compared to control subjects matched for demographic characteristics. These observations define the frame of the present study, which aims to investigate the expression level of different platelet APP isoforms using Real-Time PCR in patients affected by AD (n=20) and in age-matched controls (n= 10). Differential gene expression measurements (AD versus control) revealed a significant up-regulation for total APP (1.49-fold), for APP-KPI (1.57-fold), for APP 770 (1.37-fold), for APP 751 (1.39-fold) and for APP 695 (1.33-fold). In the present study we have demonstrated that patients with AD are characterized by differential levels of platelet-derived APP isoforms, thus suggesting that APP could be considered a potential peripheral marker for diagnosis of Alzheimer disease.

P134

Discovering mechanisms of toxicity and related biomarkers with qPCR

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One of the main aims of the VU University Amsterdam's Institute for Environmental Studies (IVM) is to contribute to solutions in the field of environmental pollution. Its position in this field is unique because of the various disciplines that are combined in our laboratory: analytical and environmental chemistry; toxicology; molecular biology; and ecology. Using an analytical chemical approach, we perform analysis of low levels of various environmental contaminants, including classical organic contaminants and emerging contaminants such as endocrine disrupting compounds. Our toxicological research focuses on determining the biological effects of (mixtures of) chemicals in the environment using both novel and classical toxicological approaches. The development of rapid and sensitive biomarkers to profile the exposure and effects of chemicals is an important pillar of our research. Quantitative RT PCR has proven to be a sensitive and rapid method to identify chemicals in the environment with specific toxic mechanisms of action. For example, we have developed qPCR assays to quantify the expression of genes involved in thyroid hormone metabolism and transport, and identified chemicals that alter expression of transport proteins and metabolic enzymes. Along these lines, we have shown for the first time that chemicals present in the blood of Arctic mammals (polar bears, seals) have the potential to induce the expression of thyroid hormone metabolizing enzymes, which may play a role in reduced thyroid hormone levels found in wild populations. Chemical analytical measurements are ongoing to reveal the identity of the chemicals likely causing this effect and help to validate the developed qPCR assays as biomarkers of thyroid hormone disruption.

P135

Evaluation of ARMS PCR when combined with limiting dilution assay for K ras mutation detection.

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Background: Different techniques have been used to detect mutant K-ras2 when it is mixed with wildtype (K-RAS2) DNA in order to diagnose pancreatic cancer at an early stage. The Amplification Refractory Mutation System (ARMS) has the potential to discriminate alleles and therefore identify point mutations and various variants of this approach have been applied. However, the results have shown poor reproducibility and in most cases have not achieved the sensitivity or specificity required for clinical use. In this study we have developed a modified ARMS technique by combining it with limiting dilution PCR in order to improve sensitivity and specificity. Limiting dilution involves diluting mixed populations of DNA and then diluting to one or two molecules per individual PCR reaction: This gives the potential to obtain individual reactions which have very high concentrations of low abundance sequences by carrying out many duplicate reactions. Materials and Methods: DNA was obtained from a cell line (PANC-1) which contained a known K-Ras mutation (G12D) and from control blood which was believed to have no mutant K-Ras (wildtype DNA, G12G). DNA was diluted to a stock concentration of 10ng/ µl. Mixtures of these DNA species were made (e.g. mix 1:10 having 1 part mutant to 10 part wildtype DNA). Pure and mixed DNA was serially diluted and PCR amplified with primers specific for G12D (aspartate primer set) or primers that were not allele specific (control primer set). Amplification was monitored in real time using the LightCycler 480 machine and at end point using gel electrophoresis. Results: The limit of detection was found to be at a dilution 0.001 for PANC-1 and 0.01 for control genomic DNA. Reproducibility was tested with both control and aspartate primer sets by repeat measures and as expected the log of dilution factor of PANC-1 DNA gave a proportional increase in crossing point (Cp) in real time PCR (linear coefficient of between 1.8 and 2.4 and R2 of between 0.82 and 0.95). However, when mix 1:10 was diluted and assayed there was an inverse relationship between log dilution factor and Cp (linear coefficient of between -0.64 and -0.62 and R2 of between 0.40 and 0.41). This means that the greater the dilution (lower actual concentration) the higher the apparent concentration appeared. Conclusion: Quantification depends on the concentration of wildtype as well as mutant K-Ras2 suggesting that the wildtype sequence is inhibitory to the mutation specific PCR. Limiting dilution will therefore give greater apparent levels of mutant sequence even before a jackpot amplification occurs and this must be taken into account when analyzing data. A dilution curve may in future replace single point analysis allowing more standard quantification of mutant sequence rather than misamplification of wildtype template.This may allow improved specificity and sensitivity of the ARMS assay for the detection of cancer.

P136

Expression of ABC transporters in multidrug resistant ovarian carcinoma cell lines using Taqman Micro Fluidic Cards

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The optimization of treatment with antitumor drugs remains a major effort of preclinical and clinical research. The over-expression of ATP binding cassette (ABC) transporters by tumor cells has been recognized as a mechanism contributing to the poor response of tumor cells to structurally and mechanistically unrelated antitumor drugs in experimental models. Whole genome approaches have documented the existence of a wide family of ABC transporters in human cells. Quantitative analysis of the expression of ABC transporters in cell lines characterized with respect to the pattern of response to clinically useful antitumor agents may be useful to define those genes that can be associated with the multidrug resistant (MDR) phenotype. In the present study, we used Taqman Micro Fluidic Cards to identify the ABC transporters transcripts that were associated with the drug-resistant phenotypes of ovarian carcinoma cell lines developed in vitro after exposure to cisplatin (IGROV-1/Pt1) or to camptothecin (IGROV-1CPT/L) and characterized by a complex pattern of resistance to multiple agents. The expression of ABC transporters in each of the two cell variants was evaluated relatively to the parental IGROV-1 cell line in two independent experiments. A statistical procedure based on multivariate approaches and re-sampling techniques was implemented to process expression values of ABC transporters. According to our analysis, a group of seven ABC transporters may be implicated in conferring multidrug resistance to the IGROV-1/Pt1 and IGROV-1CPT/L cell variants. The MDR gene signature identified in our preclinical model, throughout an ad-hoc statistical procedure, may be tested in clinical samples from ovarian cancer patients in an attempt to identify genes implicated in clinical resistance to chemotherapy.

P137

FBP1 is overexpressed in Hepatocellular Carcinoma and regulates the expression of cell-cycle and apoptosis-related genes

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In a functional yeast survival screen, we isolated a cDNA coding for a C-terminal fragment of FUSE Binding Protein 1 (FBP1) from a mamma carcinoma cDNA library. FBP1 has previously been described as a transcriptional regulator of the proto-oncogene c-myc, which is downregulated during cell differentiation. Preliminary experiments confirmed an anti-apoptotic effect of FBP1 in mammalian cells. Because FBP1 activates an important oncogene and at the same time protects cells from apoptosis, we analysed expression of FBP1 in tumors by Immunhistochemistry. We found strong nuclear staining for FBP1 in 83% (90/109) of the analysed Hepatocelluar Carcinoma, whereas in normal liver tissue no nuclear staining was observed.
Stable knockdown of FBP1 in the HCC cell line Hep3B strongly reduced tumor growth in a xenograft transplantation mouse model and we could show decreased proliferation and increased sensitivity to apoptosis for these cells in vitro.
To identify the molecular mechanism responsible for the observed effects following knockdown of FBP1, we analysed the mRNA expression of cell cycle- and apoptosis related genes by Real Time PCR. Using the Human Cell Cycle Regulation Panel, 96 (Roche), we found a significant upregulation of the cell cycle inhibitors p21 and p15 as well as downregulation of Cyclin D2. Differential expression of these genes might cause the reduction in cell

proliferation. Surprisingly, the expression level of the published FBP1 target gene c-myc was not significantly changed in these cells, indicating that regulation of c-myc expression might be independent of FBP1 in liver cells. Expression analysis of apoptosis-related genes using the Human Apoptosis Panel, 384 (Roche), revealed upregulation of the pro-apoptotic genes Bik/Nbk, Noxa, TNF-α and TRAIL. The observed changes in gene expression are in line with the observed behaviour of cells and we are currently analysing wether these differentially regulated genes are direct targets of FBP1.
Taken together, our data suggest FBP1 as a new important factor in the formation of Hepatocellular Carcinoma and we assume that the effect of FBP1 is mediated by the (direct or indirect) regulation of genes involved in the control of cell proliferation and apoptosis.

P138

Investigation of Lineage-Specific Chimerism in Post-Stem Cell Transplant Patients

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Objectives The aim of this study was to investigate the utility of lineage-specific 'chimerism' for the improved management of post-stem cell transplant patients. After transplant, an assessment of engraftment can be made by monitoring the proportions of donor and recipient cells in a sample of whole blood. Determining the mix of cells, or 'chimerism', aids the scheduling of specific therapeutic interventions. Here we extended our analysis of whole blood chimerism to a lineage-specific test, aiming to provide additional information about the dynamics of engraftment and facilitate more effective treatment in the post-transplant period.
Methodology Whole blood chimerism was measured by tracking a simplified genetic fingerprint of polymorphic short tandem repeats (STR's) that uniquely defined the donor and recipient. PCR reactions using 3 STR markers were set up using a commercially available forensics kit. The differently sized fluorescent PCR products were detected and analysed on a capillary system genetic analyser. An assessment of the proportions of donor and recipient chimerism was calculated from the analyser output using a simple algorithm measuring the peak height and area. For the lineage-specific analysis, leukocyte sub-types were isolated by cell separation using AutoMACS® immuno-magnetic separation technology. DNA was isolated from these purified fractions and levels of chimerism analysed as for whole blood.
Results Validation tests using control samples indicated chimerism could be accurately assessed in the different leukocyte lineages. Of eight patients studied, two patients displayed full donor chimerism in both the whole blood and lineage-specific analysis. Three patients with whole blood donor chimerism of <100% showed more pronounced fluctuations in lineage-specific donor chimerism. One patient, who consistently showed whole blood donor chimerism of 90-95%, displayed a similar level of donor chimerism in the myeloid fraction (CD33), but a more significant decrease to 55% in the T-cell (CD3) fraction. This patient received a stem cell transplant after refractory chronic myeloid leukaemia and so this result could give useful information as to whether the chimeric state was due to a recurrence of the original myeloid clone. Reduced T-cell chimerism is strongly associated with graft failure and low donor CD3 value could also provide evidence as to the success of the engraftment.
Conclusions This study confirmed that lineage-specific chimerism analysis represents a valuable adjunct to whole blood studies. Due to its increased sensitivity it can be used to reveal mixed chimerism in specific leukocyte populations that are masked in the whole blood analysis. Information about the relative proportions of donor and recipient T-cells is important in understanding the dynamics of engraftment and predicting graft vs leukaemia and graft vs host effects, and so T-cell chimerism represents a particularly valuable part of the analysis.

P139

Lack Of Mother-To-Newborn Transmission Of Hepatitis C Virus In Iraqi Women - A Prospective Study With Hepatitis C Virus RNA Testing

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Background: What has been published about the risk of mother-to-infant transmission of hepatitis C virus (HCV), shows variation according to the population studied and the test used. Polymerase chain reaction (PCR) was used for the first time in Iraq in a prospective study. Aims to asses the risk of vertical transmission in an unselected population of Iraqi pregnant women by usingPolymerase chain reaction (PCR) was used for the first time in Iraq in a prospective study
Material & methods: HCV antibodies (Abs) were sought with third generation enzyme immunoassay (EIA-3) in 3491 pregnant women. A positive reaction was then confirmed by a third-generation immunoblot assay (LiaTek-III). This last test was confirmed positive in 112 serum samples . we followed 26 babies of 25 anti-HCV positive mothers at first month of life. Eight of these children could be followed for six months postnataly.
Result: All the26 neonates were positive for HCV Antibodies (with EIA-3 and Lia Tek-III) during the first month of life and it completely disappeared within the following six months. HCV RNA was consistently negative in 22 sera (14 infants at first months and 8 of repeated at 6 months later) regardless of the hepatitis C virus polymerase chain reaction status of their mothers (9 of whom were positive for HCV RNA).
Cnclusion: The study showed the absence of vertical transmission of HCV from pregnant Iraqi women to their offspring.

P140

Measuring infarct size using real-time RT PCR

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Detection of necrotic tissue after ischemia is often done using 2,3,5-triphenyltetrazolium chloride (TTC). However, tissue exposed to TTC is not suitable for molecular analyses, and alternative methods for infarct size assessment are warranted. Here we report an approach for estimation of infarct size using real-time RT PCR. In short, an external RNA control gene was added to infarcted heart tissue in accordance with a recently developed protocol, and was used for real-time RT PCR normalization, giving "per wet-weight normalization". The expression of seven conventional internal RNA control genes was investigated. Of these, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was found to correlate well with TTC-measured infarct sizes, making it a potential infarct size marker. Indeed, we speculate that GAPDH expression may represent a more reliable measure of cell death than TTC. In addition, GAPDH stands out as the most suitable internal RNA control gene. In contrast, the measured amounts of ribosomal RNA (18S) and total RNA showed low correlation with infarct size, making them unsuitable for normalization of real-time RT PCR data.

P141

Oxygen restriction increases the infection potential of Listeria monocytogenes - verification of microarray chip data by quantitative real-time PCR

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Listeria monocytogenes has been implicated in several food borne outbreaks as well as sporadic cases of disease during the last two decades. Increased understanding of the biology of this organism is important in the prevention of food borne listeriosis. This is highly relevant for safety assessment of this organism in food.
We have previously shown (Andersen et al., BMC Microbiology; 2007, 7:55) that the environmental conditions to which L.

monocytogenes is exposed prior to ingestion are decisive for its in vivo infective potential in the gastrointestinal tract after passage of the gastric barrier. Infection of Caco-2 cells revealed that Listeria cultivated under oxygen-restricted conditions were approximately 100 fold more invasive than similar cultures grown without oxygen restriction. This means that not only the number of Listeria present in a given food item, but that also the physiological condition of these bacteria is important for food safety. The in vitro and in vivo data suggest that an oxygen-restricted L. monocytogenes cell represents a significantly higher risk than a cell grown without oxygen restriction.
In order to characterize genetic differences contributing to different invasiveness, microarrray gene chip technology was applied to cDNA created from RNA isolated from oxygen restricted and non-restricted cultures. The analysis confirmed several relevant genes to be differentially regulated in the two environmental conditions e.g. genes related to virulence potential of Listeria monocytogenes.
Quantitative PCR was used to verify the quantitative differences identified with the microarray chip for a selection of relevant and differentially regulated genes. We will present data, which demonstrates successful verfication of the microarray chip data, both regarding technical as well as biological replicates.

P142

Protein Thermal Shift Assays Using Applied Biosystems Real Time PCR Instruments

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The Protein Thermal Shift Assay (TSA) is a rapid and sensitive tool for monitoring protein thermostability; aiding in the identification of optimal conditions or conformations/sequences that favour protein stability, including the investigation of protein-ligand interactions. TSA is based on temperature-induced protein denaturation, monitored using an environmentally sensitive dye, such as SYPRO Orange, that is naturally quenched in a stable, aqueous solution. As the temperature increases, the protein will start to denature, exposing hydrophobic regions that will bind the SYPRO Orange dye, leading to a proportional increase in fluorescence. The increase in fluorescence over time is measured using a real-time PCR instrument. The inflection points of the resulting fluorescence/temperature plot are used to compare different test conditions, and the extent of the observed temperature shift is considered to be proportional to the affinity of a ligand to a specific protein, or representative of the stability of the protein in certain solution conditions. TSA data have been obtained from the whole range of Applied Biosystems Real Time PCR Systems; including the 7900 HT, 7500 Fast and StepOnePlusTM Real Time PCR Instruments, demonstrating the versatility of these systems. TSA has wide-ranging applications, and the use of 96- or 384-well plates lends this assay to high-throughput screening for unknown ligands and protein inhibitors in compound libraries, protein-substrate interactions, concentration-dependent stabilisation conditions, and mutational screening of protein sequences for optimization of binding stability. In addition, TSA benefits protein crystallography studies, for example, in determining the concentration of a compound that will provide maximal stability in order for a protein to reach maximum occupancy. The benefits of performing a TSA with an Applied Biosystems Real Time PCR System include the flexibility of run-method programs, catering for a range of data resolution requirements and in the use of small reaction volumes, providing fast and accurate results with only a few µg of protein.

P143

QRT- PCR in translational oncology

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Quantitative real-time PCR (qrt-PCR) technology has recently reached a level of sensitivity, accuracy and practical ease that supports its use as a routine bioinstrumentation for gene level measurement. Analysis of transcriptional activity of tumor cells or detection of tumor markers by qrt-PCR has the potential to change lung cancer diagnosis and treatment. Quantitative RT-PCR is characterized by unparalleled sensitivity and specificity, with very reliable reproducibility. Its prime advantage for gene expression analysis is its broad dynamic range of 107-fold. Moreover, it is cost-effective, feasible in every day laboratory routine and efficient in terms of biological material consumption.Knowledge of the biochemical principles underlying this biotechnology can be of great value to interpret correctly qrt-PCR data.

P144

Real-time PCR assays for Pseudo-nitzschia species in Irish waters

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Pseudo-nitzschia is a genus of marine planktonic diatoms. Some *Pseudo-nitzschia* species have the ability to produce domoic acid (DA), a toxin which can accumulate in filter-feeding shellfish tissue and cause amnesic shellfish poisoning in humans via the food chain. Monitoring Irish waters for the *Pseudo-nitzschia* genus is traditionally performed by light microscopy but this method cannot routinely identify *Pseudo-nitzschia* to species level. Species identification requires sophisticated electron microscopy which is an unsuitable technique for monitoring. Molecular methods, including real-time PCR, have the potential to rapidly identify *Pseudo-nitzschia* to species level.
We have developed real-time PCR assays targeting the rRNA ITS1 region for the rapid, high-throughput detection and identification of two *Pseudo-nitzschia* species - *P. multiseries* and *P. seriata* . These tests incorporate hybridization probes and are performed on the Roche ™ LightCycler. Specificity of each test has been verified using a broad panel of phytoplankton species and the limit of detection of each test is 10 cells in a 25 ml water sample. The real-time PCR tests have been successfully used to identify *P. multiseries* and *P. seriata* in field samples from Irish shellfish production waters. The application of these assays for quantitative detection of *P. multiseries* and *P. seriata* in field samples is under investigation.

P145

Sensitive Detection of KIT D816 in Patients with Gastrointestinal Stromal Tumours (GIST). Implication for Molecular Diagnostics and Pharmacogenomics

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Background: The pathogenic variation at codon 816 of exon 17 (D816V) in the KIT gene, occurring in some Gastrointestinal Stromal Tumours (GISTs), leads to constitutive activation of tyrosine kinase activity and confers resistance to the tyrosine kinase inhibitors, like imatinib mesylate.
The molecular recognition of this mutation, in patients with GIST, is important for determining treatment strategy but, because the ratio between malignant cells, carrying this genetic variation, and normal cell population is often small, standard detection methods can be unsuccessful. Methods: We developed high sensitivity method based on Locked Nucleic Acid (LNA) oligomers for selective detection of 2447 A>T D816V mutation of KIT gene. LNAs have been used to enhance mutant allele detection in a large excess of wild type allele, from DNA extracted by formalin-fixed paraffin-embedded of 19 GIST's biopsy. In addition we compared this result with those obtained by direct sequencing .
Results: The assay sensitivity of the method was 0.5%, assessed by serial dilution of DNA extracted from HMC-1 cell line (carrying D816V mutation), mixed up wild type DNA. By LNA assay the 57,9% (11/19) of patients were identified as carriers of mutation at codon 816, while direct sequencing was able to detect the mutation in only 2/19 (10.5%) patients. In addition, LNA assay combined to pyrosequencing platform, were able to discriminated

8 D816V point mutation in exon 17 and 3 additional pathogenic variations in short context sequence D816Y, D816N and N822Y of the 11 positive cases. Conclusion: These findings demonstrate that the LNA assay combined to Pyrosequencing is an highly sensitive and well discriminating approach for detection of KIT mutation at codon D816 and codon N822 in patients with GIST.

P146

Detection of nucleophosmin (NPM1) gene mutations with different methods in patients with AML

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A nucleophosmin (NPM1) gene encodes nuclear multifunctional proteins. Mutations – insertions of the 4 bp at the exon 12 of the NPM1 gene, located on chromosome 5q35 are the most prevalent in adult patients with acute myeloid leukemia (AML) and normal karyotype. Patients positive for mutations in NPM1 gene and negative for internal tandem duplication mutations in FMS-like tyrosine kinase 3 (FLT3) gene have favourable prognosis in this group1-4.
The aim of this study was to compare the results of the NPM1 gene mutations detection by PCR-gel detection and sequencing of the amplified PCR products. Furthermore, we want to introduce the real-time quantitative polymerase chain reaction (RQ-PCR) assay for quantitative assessment of the most frequently observed mutations (type A and B)1.
The diagnosis of AML was established followed World Health Organization classification. 95 patients with AML were included in the study. Bone marrow aspirates were used for the isolation of mononuclear cells (MC) by ficoll density centrifugation. RNA was isolated from MC by High Pure RNA Reagent Kit (Roche) and cDNA was performed by SuperScript II reverse transcriptase (Invitrogen). Amplification of the exon 12 of the NPM1 gene was performed with the forward and reverse primer as described3. The PCR products were visualized after agarose gel (4%) electrophoresis by ethidium bromide staining. All PCR- amplified samples were purified and sequenced. RQ-PCR reaction assay for type A and B mutation were performed with MutaQuantTM Standards Kit (Cancer Profiler, Ipsogen)1.
The concordance between PCR-gel and sequence detection was 100%. NPM1 mutations were identified in 22 (23%) of the 95 AML patients. The most common detected mutation type was insertion of the TCTG tetranucleotide (type A, 77%), followed by insertion of the CATG (type B, 9%), CGTG (type C, 9%), CCTG (type D, 5%) and CCAG (type NM, 5%). These are in agreement with recently published studies 1-4. Results for mutation type A and B obtained with RQ-PCR assay were in agreement with results obtained with of PCR-gel and sequence detection. At RQ-PCR reaction assay we also performed inter and intra assay for reproducibility. According to this assay variation coefficient value was below 30%, which is in agreement with literature data.
Our study shows, that PCR-gel detection is suitable screening method for identification of NPM1 mutations. A sequence detection assay is suitable for confirmation of presence mutations. RQ-PCR was found out as sensitive and reliable method. On the basis of variation coefficient was also found out that RQ-PCR method is suitable for monitoring minimal disease in AML patients.

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2 Verhaak RGW, et al. Blood, 2005; 106: 3747-3754.
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P147

Multiplex qPCR for influenza diagnostics

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The National Reference Center for Influenza monitors the circulation of influenza viruses in Germany. Diagnosis and differentiation of influenza virus subtypes is achieved by amplification of different genome segments (matrix protein M, surface proteins hemagglutinin HA and neuraminidase NA). Real-time PCR is used to accomplish a high throughput of samples.
To minimize hands-on time and financial costs, we established a multiplex real-time PCR assay for the parallel detection of Influenza A, Influenza B and an internal control (IC). Multiplex real-time PCRs were established for further differentiation of HA (H1, H3) and NA subtypes (N1, N2) of human influenza viruses. Furthermore, a multiplex real-time assay was also developed for the specific detection of important subtypes of avian influenza viruses (H5, H7, H9). All multiplex PCRs have the same specificity and detection limit as the uniplex PCRs, even though the fluorescence intensities of separate probes in the multiplex PCR are decreased in comparison to uniplex PCRs. Moreover, duplex infections only have a negligible effect on the detection limit. The PCRs were evaluated with patient specimens from the influenza seasons 2005/2006 and 2006/2007.
The results of our study confirm that multiplex PCR is a reliable tool for the detection of respiratory pathogens and, therefore, especially suitable for laboratories with high sample throughput.

P148

Detection of defective qPCR samples with Outlier of Kineret software

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Outlier is a test tool implemented within the Kineret software for real-time PCR data analysis. The aim of the test is to identify PCR samples with defective amplification. By finding and removing such samples from the analysis, Outlier improves the overall results. The computational algorithm behind the Outlier is different from recently published methods based on comparison of amplification efficiencies of samples. The unique patented method employed by Outlier utilises the principle of simultaneous recognition of several geometric characteristics, similar to that applied, for example, for the detection of fake coins in vending machines. The recognition of defective samples is based on a comparison to a reference set of well performing samples, usually the standard curve samples. The reference set is self-assed for its homogeneity prior to its use, and possibly itself corrected by excluding incompatible samples. Alternatively, where no prior assumption of good performance can be imposed on subset of samples, all samples are taken as a reference. We achieved superior performance in terms of artificially inhibited samples retrieved by the Outlier as compared to recently published methods.

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