qPCR-NGS-2013.net 18th – 22nd March 2013
Symposium & Exhibition & Workshops
Next Generation Thinking in Molecular Diagnostics
TUM, Freising-Weihenstephan, Germany

qPCR & NGS 2013 Proceedings
(online version)

6th international qPCR & NGS Symposium
Industrial Exhibition & Application Workshops

Next Generation Thinking in Molecular Diagnostics

Editor: Michael W. Pfaffl
Physiology, Freising – Weihenstephan
Weihenstephaner Berg 3
Technical University Munich (TUM)
85354 Freising
Germany

www.qPCR-NGS-2013.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 & NGS 2013 Event, the 6th International qPCR Symposium, including an Industrial Exhibition and five Application Workshops. The symposium focus is on **Next Generation Thinking in Molecular Diagnostics.** 74 lectures and 105 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 held by invited speakers, one third of the speakers will be selected from the submitted abstracts and one third of the oral contributions will be done by invited company representatives. The poster sessions will be shown from Monday evening till Wednesday early afternoon in the poster exhibition hall below the foyer.

The qPCR Event is structured in three parts:
1.   **qPCR and NGS Symposium** taking place March 18-20th, including various talk and poster sessions
2.   A parallel **Industrial Exhibition** with 40 company booth taking place March 18-20th
3.   Followed by five **qPCR and NGS Application Workshops** taking place March 21st-22nd
         powered by TATAA Biocenter Sweden, Bio-Rad, and Genomatix

The following scientific Talk and Poster sessions will be presented:
- Main Topic: Molecular Diagnostics
- Main Topic: Next Generation Sequencing (NGS)
- Main Topic: Transcriptional Biomarkers
- High-throughput Analysis in qPCR
- Single-cells Diagnostics
- MIQE & QM Strategies in qPCR
- non-coding RNAs - microRNA, siRNA, long non-coding RNAs
- Digital PCR
- Lunch Time Seminars:
    o   Biogazelle - qBASEplus qPCR Data Analysis Seminar
    o   MultiD - GenEx qPCR Data Analysis Seminar
    o   Life Technologies - digital PCR Seminar
    o   Genomatix - NGS data analysis Seminar

The scientific organization is managed by international well-known scientists in the field of molecular diagnostics:
Stephen Bustin   Prof. of Allied Health and Medicine, Faculty of Health, Anglia Ruskin University, UK
Mikael Kubista   Prof. of Biotechnology, BTU, Czech Academy of Sciences & TATAA Biocenter, Sweden
Jo Vandesompele   Prof. at the Center of Medical Genetics, University of Ghent, Belgium
Michael W. Pfaffl   Prof. of Molecular Physiology, TUM Weihenstephan, Germany   (scientific coordination)

The event organization will be managed by Sylvia Pfaffl, bioMCC, Germany.

The qPCR & NGS 2013 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 poster sessions, the industrial exhibition, the application workshops and the social program during the following week.

Best regards

Scientific coordinator

Event Agenda Overview

Online agenda HTML => http://agendaHTML.qPCR-NGS-2013.net   online agenda PDF => http://agendaPDF.qPCR-NGS-2013.net

	Lecture hall no. 14 (HS 14)	Lecture hall no. 15 (HS 15)	Foyer & Seminar rooms S1 & S2
Sunday **17th Mach**			12:00 – 18:00 **Industrial Exhibition Built up**
			15:00 – 18:00 **Arrival & Registration**
Monday **18th March**	10:00 – 10:15 **Opening of the Symposium** Welcome by Michael W. Pfaffl		8:00 – 10:00 **Arrival & Registration** 8:00 – 10:00 **Poster Setup** in Foyer lower level
	10:15 – 13:00 **Molecular Diagnostics 1**		10:00 – 21:00 **Industrial Exhibition** in Foyer
	13:00 – 14:00 **Lunch**		
	14:00 – 18:00 **Next Generation Sequencing 1**		
	18:00 – 22:00 **Reception in the Industrial Exhibition**		18:00 – 22:00 **Evening Poster Session** in Foyer lower level
Tuesday **19th March**	8:30 – 12:30 **Digital PCR**	8:30 – 12:30 **Next Generation Sequencing 2**	8:30 – 14:00 **Industrial Exhibition** in Foyer
	12:30 – 14:00 **Lunch**		12:30 – 14:00 **Lunch Poster Session** in Foyer lower level
	13:00 – 14:00 **Life Technologies:** **digital PCR lunch time seminar**	13:00 – 14:00 **Biogazelle: qbasePLUS** **data analysis lunch time seminar**	
	14:00 – 18:30 **Transcriptional Biomarkers**	14:00 – 18:30 **High Throughput Analysis &** **qPCR optimization**	14:00 – 18:00 **Industrial Exhibition** in Foyer
	19:30 – 24:00 **Symposium Gala Dinner** Location: Bräustüberl Weihenstephan, Freising International - Bavarian - European Buffet, Music & Dancing & Cocktails		
Wednesday **20th March**	8:30 – 13:00 **MIQE & QC in qPCR**	8:30 – 13:00 **Non-coding RNAs**	8:30 – 14:00 **Industrial Exhibition** in Foyer
	12:30 – 14:00 **Lunch**		12:30 – 14:00 **Lunch Poster Session** in Foyer lower level
	13:00 – 14:00 **Genomatix: NGS** **data analysis lunch time seminar**	13:00 – 14:00 **MultiD: GenEx** **qPCP data analysis lunch time seminar**	
	14:00 – 14:15 **Best Academic Poster Award**		
	14:15 – 16:45 **Single-Cell Diagnostics**	14:15 – 16:45 **Molecular Diagnostics 2**	14:00 – 16:30 **Poster Take Down** in Foyer lower level
	16:45 – 17:00 **Closing of the Symposium** Michael W. Pfaffl		
Thursday **21st March** **Friday** **22nd March**	**Seminar rooms S1 - S3 & Computer seminar rooms PU26 and PU26A** 9:00 - 17:00 **qPCR & NGS Application Workshops:** • **Basic real-time qPCR Application Workshop** (2-days) hosted by TATAA Biocenter • **Experimental design and statistical data analysis for qPCR** (2-days) hosted by TATAA Biocenter • **MIQE: Quality control of qPCR in Molecular diagnostics** (2-days) hosted by TATAA Biocenter • **digital PCR** (2-days) hosted by Bio-Rad • **NGS data analysis workshop** (2-days) hosted by Genomatix		

Agenda qPCR & NGS 2013 Event

Sunday 17th March 2013

12:00 – 18:00	Built-up for Industrial Exhibition
15:00 – 18:00	Arrival & Registration Poster Setup

Monday 18th March 2013

Welcome & Opening of the Symposium
Lecture hall   HS 14

08:00 – 10:00	Built-up for Industrial Exhibition Arrival & Registration
09:00 – 10:00	**Welcome Coffee & Tea**
10:00	**Welcome & Opening of the Symposium** Michael W. Pfaffl Scientific coordinator of the qPCR & NGS Event

Molecular diagnostics session 1

Time: Monday, 18/03/2013: 10:15am – 13:15pm
Location: Lecture hall 14
Session Chair: Michael W Pfaffl, Mikael Kubista

10:15 **Near-Oncology Patient (and Near-Oncology Health Care Provider) Molecular Testing on the GeneXpert**
<u>Russell Higuchi</u>
Cepheid Fellow R&D, Cepheid, Sunnyvale, CA, USA

10:45 **High throughput mRNA and protein expression profiling by qPCR**
<u>Mikael Kubista</u>[1,2], Robert Sjöback[1], Jens Björkman[1], David Svec[1], Anders Stahlberg[1,4], Vendula Rusnakova[2], Miroslava Anderova[3]
[1]TATAA Biocenter, Sweden; [2]Institute of Biotechnology, Czech Academy of Sciences; [3]Institute of Experimental Medicine, Czech Academy of Sciences; [4]Cancer center, University of Gothenburg

11:15 **Sequence, Shape, Function: Synthetic Biology by DNA**
<u>Hendrik Dietz</u>
Technische Universität München, Germany

11:45 **The New LightCycler® 96 System: It Is So Easy To Be A Lab Hero**
<u>Ralf Peter Mauritz</u>
Roche Diagnostics GmbH, Germany

12:15 **A Molecular Assay With Laser-heated Nanoparticles**
<u>Lars Ullerich</u>
GNA Biosolutions GmbH, Germany

12:45 **Going to the limits of Multiplex Real-time PCR**
<u>Olfert Landt</u>[1], Ulrich Lass[1], Matthias Ballhause[1], Johannes Kusters[2], Pranav Patel[3]
[1]Tib Molbiol Syntheselabor GmbH, Berlin, Germany;[2] Medical Microbiology, University Medical Center Utrecht, The Netherlands;[3]Robert-Koch-Institut, Berlin, Germany

13:00 – 14:00	**Lunch in the student cafeteria**

NGS 1 - Next Generation Sequencing session 1

Time: Monday, 18/03/2013: 2:00pm - 6:00pm
Location: Lecture hall 14
Session Chair: Vladimir Benes, Alexander Schramm

14:00 **RNA-Seq: opportunities, limitations and applications in cancer research**
<u>Alexander Schramm</u>[1], Marcel Martin[2], Johannes H. Schulte[1], Johannes Köster[2], Pieter Mestdagh[3], Jo Vandesompele[3], Sven Rahmann[2,4]
[1]University Hospital Essen, Pediatric Oncology, Germany; [2]TU Dortmund, Dept. of Computer Science, LS11, Germany; [3]Center for Medical Genetics Ghent, Belgium; [4]University Hospital Essen, Genome Informatics, Germany

14:30 **Advancements in Ion Torrent RNA Sequencing: More and Less**
<u>Richard Fekete</u>, Kelli Bramlett, Yongming Sun, Jeff Schageman, Luming Qu, Ross Hershorn, Charmaine San Jose Hinahon, Brian Sanderson, Angie Cheng, Bob Setterquist
Life Technologies, United States of America

15:00 **The Potential for Next Generation Sequencing in Forensics**
<u>Nicola Oldroyd</u>
R&D, Illumina, United Kingdom

15:30 – 16:00	**Coffee break & Networking**

16:00 **Advances in NGS library preparation – the devil is in the detail**
Bianka Baying, Bettina Haase, Jonathon Blake, Dinko Pavlinic, Jürgen Zimmermann, <u>Vladimir Benes</u>
EMBL-GeneCore, Meyerhofstr. 1, Heidelberg, Germany

16:30 **The NeXT generation Variant annotation Tracker: a one stop cloud solution to exome sequencing data analysis**
<u>Bram De Wilde</u>, Tom Sante, Jasper Anckaert, Jan Hellemans, Frank Speleman, Björn Menten, Jo Vandesompele
Center for Medical Genetics, Ghent University, De Pintelaan, Gent, Belgium

17:00 **Transforming NGS for clinical research and diagnostics**
<u>Erik Söderbäck</u>
Market Development Manager, Qiagen, Germany

17:30 **New developments in NGS target enrichment**
<u>Götz Frommer</u>
Sales Manager Genomics Germany, Agilent Technologies Diagnostics and Genomics Group, Germany

18:00 – 22:00	**Evening Poster Session**
18:00 – 22:00	**Networking** **Reception in Industrial Exhibition**

Tuesday 19th March 2013

Digital PCR

Time: Tuesday, 19/03/2013: 8:30am - 12:30pm
Location: Lecture hall 14
Session Chair: Jim Francis Huggett, Ariane De Ganck

8:30 **An introduction to digital PCR**
Jim Francis Huggett
LGC, United Kingdom

9:00 **Performance Of A Next Generation Fixed Microwell Platform For Digital PCR**
David N. Keys
Life Technologies, United States of America

9:30 **Quantitative Detection of Cancer Biomarkers in Picoliter Droplets.**
Valerie Taly[1,3], **Deniz Pekin**[1,3], **Corinne Normand**[1], **Zakaria El Harrak**[1], **Thevy Hor**[1], **Li Xinyu**[2], **Ivan Atochin**[2], **Steve Kotsopoulos**[2], **Delphine Le Corre**[1], **Leonor Benhaim**[1], **J. Brian Hutchison**[2], **Darren R. Link**[2], **Helene Blons**[1], **Philippe Nizard**[1], **Pierre Laurent-Puig**[1]
[1]Université Paris Descartes; INSERM; Centre Universitaire des Saints-Pères, France; [2]RainDance Technologies, Lexington, USA; [3]Université de Strasbourg; CNRS UMR 7006, Strasbourg Cedex, France

10:00 – 10:30 **Coffee break & Networking**

10:30 **Enhanced Resolution of Copy Number Variants in Domestic Animal Species**
Jennifer Meadows
Uppsala University, Sweden

11:00 **RainDrop Digital PCR: Single Molecule Counting With A Droplet Digital PCR Platform**
Adam Corner
RainDance Technologies, United States of America

11:30 **Use Of Digital PCR For Improved Copy Number Quantification**
Ariane De Ganck[1], **Annelies Dheedene**[2], **Björn Menten**[2], **Jan Hellemans**[1], **Jo Vandesompele**[1]
[1]Biogazelle, Zwijnaarde, Belgium; [2]Center for Medical Genetics, Ghent University, Ghent, Belgium

12:00 **Droplet Digital PCR For Free Fetal DNA Analysis: Statistical Modelling And Evaluation For Non-Invasive Prenatal Diagnosis Of An X-linked Deletion.**
Emmanuel Debrand[1], **Michael Samuels**[2], **Sarah Clinton**[1], **Stephanie Allen**[1]
[1]Birmingham Women's Hospital, United Kingdom; [2]RainDance Technologies

12:30 – 14:00 **Lunch in the student cafeteria**

12:30 – 14:00 **Lunch Poster Session**

Life Technologies: digital PCR lunch time seminar

Time: Tuesday, 19/03/2013: 1:00pm - 2:00pm
Location: Lecture hall 14

13:00 **Digital PCR Workshop with the QuantStudio 3D**
David Keys
Life Technologies, United States of America

Transcriptional Biomarkers

Time: Tuesday, 19/03/2013: 2:00pm - 6:30pm
Location: Lecture hall 14
Session Chair: Stephen Bustin, Massimo Bionaz

14:00 **Systems Physiology in Cattle: Transcriptome Dynamics and Beyond**
Massimo Bionaz[1], **Juan J Loor**[2]
[1]Oregon State University, United States of America; [2]University of Illinois at Urbana-Champaign

14:30 **An organotypic culture model for the study of colorectal cancer**
Stephen Bustin
Postgraduate Medical Institute, Anglia Ruskin University, Chelmsford, UK

15:00 **Ion AmpliSeq[TM] Technology: A Tool For Biomarker Discovery And Beyond**
David Ruff
Life Technologies, United States of America

15:30 **A Kinetic PCR Model Function and its Application on the LightCycler® 96**
Rolf Knobel
Roche Diagnostics International, Switzerland

16:00 – 16:30 **Coffee break & Networking**

16:30 **Transcriptional Profiling To Address Molecular Determinants Of Endometrial Receptivity – Lessons From Studies In Livestock Species**
Susanne E. Ulbrich[1], **Anna E. Groebner**[1], **Stefan Bauersachs**[2]
[1]Physiology Weihenstephan, Technische Universität München, Freising, Germany; [2]Laboratory for Functional Genome Analysis (LAFUGA), Gene Center, LMU Munich, Munich, Germany

17:00 **Candidate Blood Transcriptomic Markers of Early Onset Major Depression Derived from Etiological Animal Models of Depression**
Eva E. Redei[1], **William Gardner**[2,3], **Andrea Luis**[2], **Brandon Strange**[3], **Kathleen Pajer**[2]
[1]Northwestern University Feinberg School of Medicine, United States of America; [2]Department of Psychiatry, Dalhousie University Faculty of Medicine, Canada; [3]Department of Pediatrics, Ohio State University College of Medicine, Columbus, OH, USA

17:30 **Novel Solution Enables Automation of Data Analysis and QC of Real-Time PCR Diagnostics**
Aron Cohen, Ze'ev Russak, Martine Bernstein
Azure PCR, United Kingdom

18:00 **A workflow for the isolation and molecular characterization of Individual Circulating Tumor Cells (CTCs) to enable cell heterogeneity analysis and personalized therapy**
Gianni Medoro, **Francesca Fontana**, Alex Calanca, Chiara Bolognesi, Stefano Gianni, Maximilian Sergio, Giulia Bregola, Anna Doffini, Giulio Signorini, Antonino Catania, Manuela Banzi, Elena Peruzzi, Giuseppe Giorgini, Nicolò Manaresi
Silicon Biosystems, Italy

19:30 – 24:00 ## Symposium Gala Dinner

Location: Bräustüberl Weihenstephan, Freising International – Bavarian - European- Buffet Music & Dancing & Cocktails

NGS 2 - Next Generation Sequencing session 2

Time: Tuesday, 19/03/2013: 8:30am - 12:30pm
Location: Lecture hall 15
Session Chair: Robert P. Loewe, Michael W Pfaffl

8:30 **The Road to Genomic Medicine is Paved with Data and Information**
John Quackenbush
Dana-Farber Cancer Institute, United States of America

9:00 **Sequencing the "Unsequenceable" Single-Molecule, Real-Time (SMRT™) DNA Sequencing: Technology Overview and Recent Applications**
Ralph Vogelsang
Pacific Biosciences, United States of America

9:30 **Droplet Digital PCR and Next Gen Sequencing: Digital Biology in High Definition**
Svilen Tzonev
Bio-Rad, United States of America

10:00 **The WaferGen's SmartChip System: "Cycling in the world of high- throughput qPCR and target enrichment"**
Stefaan Derveaux
WaferGen Biosystems Europe S.à r.l., Luxembourg

10:30 – 11:00 **Coffee break & Networking**

11:00 **The distribution of small RNAs in milk and their functional relation to mammary gland physiology**
Michael W Pfaffl[1], Benedikt Kirchner[1], Alexander Hahn[2], Vladimir Benes[3]
[1]Physiology Weihenstephan, Technical University Munich, Germany; [2]Genomatix Software GmbH, Munich, Germany; [3]EMBL Genomics Core Facility (GeneCore), Heidelberg, Germany

11:30 **Engineered Enzymes and Optimized Workflows for Next Generation Sequencing**
Eric van der Walt, Maryke Appel, Gavin Rush, John Foskett, Paul McEwan
Kapa Biosystems, Woburn, MA, USA

12:00 **Comprehensive Biomarker Approach By Utilizing qPCR, NGS, in situ PCR: A Tool Box To Decipher Cancer**
Robert P. Loewe
GeneWake GmbH, Germany

12:30 **Less Than 80% Consensus On The Same Data - Comparative Variant Calling Of Pipelines And Replicates**
Lu Zhang[1], Milena Kovacevic[2], Milos Popovic[2], **Sebastian Wernicke[1]**
[1]Seven Bridges Genomics, Cambridge, MA, USA; [2]Seven Bridges Genomics, Belgrade, Serbia

12:30 – 14:00 **Lunch in the student cafeteria**

12:30 – 14:00 **Lunch Poster Session**

Biogazelle qbasePLUS: data analysis lunch time seminar

Time: Tuesday, 19/03/2013: 1:00pm - 2:00pm
Location: Lecture hall 15

13:00 **qbasePLUS speeds up the analysis of your qPCR data and improves the accuracy of your results**
Barbara D'haene
Biogazelle, Ghent, Belgium

High Throughput Analysis & qPCR Optimisation

Time: Tuesday, 19/03/2013: 2:00pm - 6:30pm
Location: Lecture hall 15
Session Chair: Jan Hellemans, Patricia de Winter

14:00 **Insights from the first RT-qPCR based human transcriptome profiling based on wet lab validated assays.**
Jan Hellemans, Pieter Mestdagh, Barbara D'haene, Ariane Deganck, Jo Vandesompele
Biogazelle, Belgium

14:30 **Overcoming PCR inhibition: Next Generation qPCR and RT-qPCR ToughMix® Reagents for High Sensitivity Quantification and Accurate Genotyping from Crude Samples.**
David Mark Schuster
Quanta BioSciences, United States of America

15:00 **Rapid qPCR using a novel Taq mutant**
Patricia de Winter[1], David Sugden[2]
[1]UCL, United Kingdom; [2]KCL, United Kingdom

15:30 **A Nū Solution for Real-Time Quantitative PCR**
Gothami Padmabandu
Illumina Inc, United States of America

16:00 – 16:30 **Coffee break & Networking**

16:30 **Twisted Intercalating Nucleic Acid (TINA) – a novel group of molecules with improved performance in PCR and qPCR applications**
Rainer Schubbert
Eurofins Medigenomix, Germany

17:00 **Automated PCR Setup for High Throughput Analysis Enabled by the Labcyte Echo® 525 Liquid Handler and Access™ Workstation**
Celeste Glazer, Carl Jarman
Labcyte, United States of America

17:30 **Factors influencing the transfer of multiplex assays between qPCR instruments**
Ossian Saris
Thermo Fisher Scientific, Vantaa, Finland

18:00 **ValidPrime questions the need for DNase treatment in RT-qPCR experiments**
Henrik Laurell[1], Jason Iacovoni[1], Jean-José Maoret[1], Jean-François Arnal[1], Mikael Kubista[2]
[1]Inserm / Université Paul Sabatier UMR1048, Institut des Maladies Métaboliques et Cardiovasculaires (I2MC), BP84225, 31432 Toulouse cedex 4, France; [2]TATAA Biocenter AB, Göteborg, Sweden

19:30 – 24:00 ## Symposium Gala Dinner

Location: Bräustüberl Weihenstephan, Freising
International – Bavarian - European- Buffet
Music & Dancing & Cocktails

Wednesday 20th March 2013

MIQE & QC strategies in qPCR

Time: Wednesday, 20/03/2013: 8:30am – 13:00pm
Location: Lecture hall 14
Session Chair: Gregory L Shipley, Afif Michel Abdel Nour

8:30 **MIQE 2009-2013 - its impact four years after publication**
Stephen Bustin
Postgraduate Medical Institute, Anglia Ruskin University, Chelmsford, UK;

9:00 **Quality control in Quantitative PCR**
Kristina Lind, Jennifer Pettersson, Robert Sjöback, Mikael Kubista
TATAA Biocenter, Sweden

9:30 **Applying the MIQE guidelines to clinical and pre-clinical trials**
Maxime Dooms[2], Abalo Chango[2], Essam Azhar[1], Steve Harakeh[1], Elie Barbour[3], Flore Depeint[2], **Afif Michel Abdel Nour**[1]
[1]KAU/KFRMC/ Special Infectious Agent unit Biosafety Level 3, SA; [2]Institut Polytechnique LaSalle Beauvais, France; [3]American University of Beirut, Lebanon

10:00 **Applying the MIQE Guidelines to Screens Utilizing qPCR Focused Arrays**
Gregory L Shipley
Shipley Consulting, LLC, United States of America;

10:30 – 11:00 **Coffee break & Networking**

11:00 **Management and Automation of qPCR Diagnostic Workflows**
Matjaz Hren
BioSistemika, Slovenia

11:30 **The use and usefulness of amplification curve analysis in quantitative PCR.**
Jan M Ruijter[1], Michael W Pfaffl[2], Sheng Zhao[3], Andrej N Spiess[4], Gregory Boggy[5], Jochen Blom[6], Robert G Rutledge[7], Davide Sisti[8], Antoon Lievens[9], Katleen De Preter[10], Stefaan Derveaux[11], Jan Hellemans[12], Jo Vandesompele[10]
[1]Academic Medical Centre, Amsterdam, NL; [2]Technical University of Munich, Weihenstephan, D; [3]University of California, Berkeley, USA; [4]University Hospital Hamburg-Eppendorf, D; [5]eDNA Software Inc., Ann Arbor, USA; [6]Center for Biotechnology, Bielefeld University, D; [7]Laurentian Forestry Centre, Quebec, CA; [8]University of Urbino, Urbino, Italy; [9]Department of Applied Mathematics and Computer Science, Ghent, BE; [10]Center for Medical Genetics, Ghent, Belgium; [11]Wafergen, Fremont, CA, USA; [12]Biogazelle, Zwijnaarde, BE

12:00 **Four Years of RDML qPCR Data Format – Achievements and Opportunities**
Andreas Untergasser[1], Steve Lefever[2], Jan M Ruijter[3], Jan Hellemans[4], Jo Vandesompele[2,4]
[1]University Heidelberg, Heidelberg, Germany; [2]Ghent University, Ghent, Belgium; [3]Academic Medical Center, Amsterdam, The Netherlands; [4]Biogazelle, Zwijnaarde, Belgium

12:30 **The Impact of MIQE Guidelines in the Plant Science Community.**
Ellen De Keyser, Laurence Desmet, Jan De Riek
ILVO, Belgium

12:30 – 14:00 **Lunch in the student cafeteria**

12:30 – 14:00 **Lunch Poster Session**

Genomatix: NGS data analysis lunch time seminar

Time: Wednesday, 20/03/2013: 1:00pm - 2:00pm
Location: Lecture hall 14

13:00 **Start making sense - NGS data analysis with Genomatix**
Christian Zinser
Genomatix Software GmbH, Munich, Germany

Best Academic Poster Award

Time: Wednesday, 20/03/2013: 2:00pm - 2:15pm
Location: Lecture hall 14
Session Chair: Michael Pfaffl, Klemen Zupancic

14:00 Award for the best academic poster in the qPCR & NGS Poster presentations

Single-Cell Diagnostics

Time: Wednesday, 20/03/2013: 2:15pm - 4:45pm
Location: Lecture hall 14
Session Chair: Kenneth James Livak, Anders Ståhlberg

14:15 **A Microfluidic Device that Isolates Single Cells then Processes RNA for qPCR or Sequencing**
Kenneth James Livak
Fluidigm Corporation, United States of America

14:45 **Genome Analysis Of Individual Cells**
Christian Korfhage
QIAGEN GmbH, Germany

15:15 **Quantitative PCR Analysis of DNA, RNAs, and Proteins in the Same Single Cell**
Anders Ståhlberg
University of Gothenburg, Sweden

15:45 **Visualizing gene expression at the single cell, single chromosome, single RNA, and single base level**
Marshall Levesque, Arjun Raj
Biosearch Technologies Inc., United States of America

16:15 **Single-Cell Digital Gene Expression On Up To 800 Unique Transcripts Using Optically-Barcoded Single-Nucleic Acid Counting: Comparison With Microfluidic qPCR And RNA-seq (Whole Transcriptome)**
Michael Rhodes
Nanostring Technologies, United Kingdom

Non-coding RNAs
microRNA, siRNA and long non-coding RNAs

Time: Wednesday, 20/03/2013: 8:30am – 1:00pm
Location: Lecture hall 15
Session Chair: Pieter Mestdagh, Mirco Castoldi

8:30 **Evaluation of quantitative microRNA gene expression platforms in the microRNA Quality Control (miRQC) study**

Pieter Mestdagh[1], Toumy Gettouche[2], Thomas Peters[3], Nicole Hartmann[3], <u>Jo Vandesompele</u>[1]

[1]Ghent University / Biogazelle, Belgium; [2]University of Miami, Florida, USA; [3]Novartis Institutes for BioMedical Research, Novartis, Basel, Switzerland

9:00 **Non-Coding RNAs in Tumor and Inflammatory Diseases.**

<u>Jörg Hackermüller</u>[1,2,3], **The Ribolution Project Consortium**[4]

[1]Young Investigators Group Bioinformatics and Transcriptomics, Helmholtz Centre for Environmental Research - UFZ, [2]RNomics group, Fraunhofer IZI, [3]Department of Computer Science, University of Leipzig, Germany

9:30 **An Optimized miRNA Profiling System for Limiting Samples**

<u>Jonathan Michael Shaffer</u>

QIAGEN, United States of America;

10:00 **Emerging role of blood circulating microRNA as non-invasive biomarker**

<u>Mirco Castoldi</u>

Universitätsklinikum Düsseldorf, Germany

10:30 – 11:00 **Coffee break & Networking**

11:00 **Generating Robust Results From qPCR Analysis Of MicroRNAs In Biofluids**

<u>Ditte Andreasen</u>, Thorarinn Blondal, Maria Wrang Theilum, Niels Tolstrup, Jörg Krummheuer, Nana Jacobsen, Peter Mouritzen

Exiqon A/S, Denmark

11:30 **Integration of disparate sources of information to predict miRNA-mRNA interactions**

Ander Muniategui*[1], Ignacio Sanchez-Caballero*[2], Rubén Nogales-Cadenas[2], Carlos O. Sánchez-Sorzano[2], Alberto Pascual-Montano*[2], <u>Angel Rubio</u>*[1]

[1]CEIT & TECNUN, University of Navarra, Spain; [2]Funciotnal Bioinformatics group, CNB-CSIC, Madrid, Spain

12:00 **How to Narrow Down the Complexity of Possible miRNA and mRNA Interactions in Cellular Differentiation?**

<u>Swanhild U Meyer</u>[1], Steffen Sass[2], Fabian J Theis[2], Michael W Pfaffl[1]

[1]Physiology Weihenstephan, ZIEL Research Center for Nutrition and Food Sciences; [2]MIPS, Institute for Bioinformatics and System Biology, Helmholtz Center Munich, German Research Center for Environmental Health, Neuherberg, Germany

12:30 **High-throughput lncRNA expression profiling identifies candidate cancer lncRNAs**

<u>Pieter Mestdagh</u>[1], Steve Lefever[1], Kristina Althoff[2], Carina Leonelli[1], Jan Hellemans[3], Marine Jean-Christophe[4], Johannes Schulte[2], Jo Vandesompele[1]

[1]Center for Medical Genetics, Ghent University, Belgium; [2]Department of Pediatric-Oncology, University Hospital Essen, Germany; [3]Biogazelle, Ghent, Belgium; [4]VIB Laboratory for Molecular Cancer Biology, Leuven, Belgium

12:30 – 14:00 **Lunch in the student cafeteria**

12:30 – 14:00 **Lunch Poster Session**

GENEX: qPCR data analysis lunch time seminar

Time: **Wednesday, 20/03/2013: 1:00pm - 2:00pm**
Location: **Lecture hall 15**

13:00 **GenEx - the ultimate tool for qPCR data analysis**

<u>Mikael Kubista</u>

MultiD, TATAA Biocenter, Sweden

Molecular Diagnostics session 2

Time: **Wednesday, 20/03/2013: 2:15pm - 4:45pm**
Location: **Lecture hall 15**
Session Chair: **Steve Lefever, Irmgard Riedmaier**

14:15 **Cost-effective and robust genotyping using double-mismatch allele-specific quantitative PCR**

<u>Steve Lefever</u>[1], Ali Rihani[1], Filip Pattyn[1], Tom Van Maerken[1], Jan Hellemans[2], Jo Vandesompele[1,2]

[1]Center for Medical Genetics Ghent, Ghent University, Ghent, Belgium; [2]Biogazelle, Zwijnaarde, Belgium

14:45 **Assessment of Transcriptional Activity of *Borrelia burgdorferi* and Host Cytokine Genes During Early and Late Infection in a Mouse Model**

<u>Emir Hodzic</u>

University of California at Davis, United States of America

15:15 **Is lung cancer genetic heterogeneity responsible for resistance to EGFR tyrosine kinase inhibitors? Contribution of digital PCR**

<u>Pascale Tomasini</u>, Veronique Secq, Isabelle Nanni, Antoine Carlioz, Fabrice Barlesi, L'Houcine Ouafik, Frederic Fina

Assistance Publique Hôpitaux de Marseille, France

15:45 **Cost-effective real-time analysis by mediator probe (RT-) PCR**

<u>Simon Wadle</u>[1], Stefanie Rubenwolf[1], Michael Lehnert[1], Bernd Faltin[2], Roland Zengerle[1,3,4], Felix von Stetten[1,3,4]

[1]Laboratory for MEMS Applications, IMTEK - Department of Microsystems Engineering, University of Freiburg, Georges-Koehler-Allee 103, 79110 Freiburg, Germany; [2]Robert Bosch GmbH, Applied Research 1 - Microsystem Technologies - Microstructuring and Assembly, Postfach 10 60 50, 70049 Stuttgart, Germany; [3]HSG-IMIT - Institut für Mikro- und Informationstechnik, Georges-Koehler-Allee 103, 79110 Freiburg, Germany; [4]BIOSS - Centre for Biological Signalling Studies, University of Freiburg, 79110 Freiburg, Germany

16:15 **Somatic mutations – detecting less than 0.05% KRAS and BRAF mutation in a background of wildtype DNA**

Christina Andersen[1], Tine Y. Wollff[2], Alice Riva[3], Milo Frattini[3], <u>Ulf Bech Christensen</u>[1], Majbritt H. Kyneb[2]

[1]PentaBase ApS, Lumbyvej 11, building 5V, 5000 Odense C, Denmark; [2]Danish technological institute, Life Science department, Kongsvang Allé 29, 8000 Aarhus C, Denmark; [3]Laboratory of Molecular Pathology, Institute of Pathology Via in Selva, 24 6600 Locarno, Switzerland;

Closing of the Symposium
Lecture hall   HS 14

16:30 **Closing of the Symposium & Farewell**

Michael W. Pfaffl

Thursday 21st March & Friday 22nd March 2013

qPCR Application Workshops

The workshops are aimed at giving participants a deep and objective understanding of real-time quantitative PCR, digital PCR, biostatistics, expression profiling, and its applications. The courses are intended for academic or industrial persons considering working with quantitative PCR 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 15 participants (biostatistics 30 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.

All workshops start on **Thursday and Friday at 9 am until 5 pm**. Three workshops are hosted by the TATAA Biocenter Sweden, (www.tataa.com) and the digital PCR workshop is hosted by Bio-Rad (www.Bio-Rad.com). The qPCR workshop seminar rooms, S1, S2, S3 and computer seminar rooms PU26 and PU26A (fully equipped with computers) are close to the central lecture hall.

qPCR Workshop topics:

- **Basic Module qPCR Application Workshop (2-days)** hosted by TATAA   Seminar room **S3**
- **Experimental design and statistical data analysis for qPCR (2-days)** hosted by TATAA   Computer seminar room – **PU26**
- **MIQE: Quality control of qPCR in Molecular diagnostics (2-days)** hosted by TATAA   Seminar room **S2**
- **Digital PCR   (2-days)** hosted by Bio-Rad   Seminar room **S1**

NGS data analysis workshop:

The NGS data analysis workshop on 21st and 22nd March is hosted by **Genomatix** (www.Genomatix.com)

- **NGS data analysis workshop** (2-days) hosted by Genomatix   Computer seminar room **PU26A**

Basic Module qPCR Application Workshop (2-days)   Seminar room S3

Description: The basic real-time qPCR course. You will aquire a comprehensive overview of the possibilities with real-time PCR, how to use it and how to analyze the results. The course contains:

Day 1
- Basic PCR and qPCR
- Review of different detection technologies (SYBR Green I, hydrolysis probes, Molecular Beacons, etc)
- Different instrument platforms
- Applications and possibilities of qPCR.
- Primer and probe design
- Basic data handling and analysis
- Experimental design and optimization

Day 2
- Introduction to quantification principles
- Quantification strategies, uses and limitations
- Strategies for normalization of qPCR data
- Calculations using different relative quantification methods
- Absolute quantification
- Validation of qPCR assays

Experimental design and statistical data analysis for qPCR (2-days)   Computer seminar room PU26

Description: Learn how appropriate statistics shall be selected and applied correctly to get the most out of your qPCR data. The course includes theoretical lectures combined with practical data analysis performed with qPCR analysis software. The course contains:

Day 1 - Statistical analysis of real-time PCR data
- Basic principles of statistics
- Advanced principles of statistics
- Statistical tests
- Ability to detect a difference

Day 2 - Gene expression profiling with real-time PCR
- Multiplate measurements
- Standard curves and absolute quantification
- Experimental design, Selecting reference genes
- Relative quantification, Comparison of groups
- Expression profiling

The computer seminar room PU26 is fully equipped with computers. In the qPCR data analysis workshop the data conversion, normalisation procedure, biostatistical calculations and the expression profiling will be done with the **newest GenEx software by MultiD**.

Download a free GenEx trial version => Genex.gene-quantification.info

MIQE: Quality control of qPCR in Molecular diagnostics (2-days)   Seminar room S2

This course will go deep into the MIQE guidelines; describe the important steps in qPCR and how you should work to fulfill the guidelines. The course will also focus on how you do proper quality control of your qPCR assays to be used in molecular diagnostics. It will describe which controls that are needed and the statistics on how to do the evaluations.

The course contains:
- Introduction to the MIQE guidelines
- Nucleic acid extraction and quality control
- Reverse transcription
- Primer design
- qPCR protocol and validation, LOD, LOQ
- Principles of statistics
- Normalization
- Relative quantification
- Absolute quantification
- Variance contribution, experimental design
- Precision testing

digital PCR (2-days)   Seminar room S1

Description: Learn how to plan, perform, and analyze digital PCR experiments and how digital PCR can help your research to overcome the limitations of real-time qPCR.

Day 1
- Welcome and introductions
- Introduction to Digital PCR
- Droplet generation and PCR start for CNV experiment
- ddPCR applications: CNV
- Start DR for CNV experiment
- Droplet generation and PCR start for RED and ABS experiments
- ddPCR Applications: RED and ABS
- CNV results analysis
- Start DR for RED/ABS experiment
- Review of the day

Day 2
- ddPCR: basic statistics
- RED/ABS results analysis
- Other ddPCR Applications: gene expression and NGS
- When qPCR and when ddPCR? Moving from qPCR to ddPCR
- Open Q&A session
- Review of the workshop

NGS data analysis workshop (2-days)   Computer seminar room PU26A

Description: The large amounts of data derived from next generation sequencing projects makes efficient data mining strategies necessary. In the course you will learn strategies for the analysis of different kinds of next generation sequencing data. The workshop is based on real world examples and will use the Genomatix software, which provides a graphical user interface; no programming, scripting, or command line tool knowledge is necessary to attend. The computer seminar room PU26A is fully equipped with computers.

Day 1 - General introduction to the Genomatix system
- **SNP analysis:**
  - GMS demo: mapping of DNA data and SNP detection
  - GGA hands-on: SNP effects analysis characterization of regulatory SNPs
- **CNV analysis:**
  - GGA hands-on: pairwise CNV analysis
- **Methylation analysis:**
  - GMS demo: bisulfite mapping and methylation analysis
 - GGA hands-on: visualization of methylation data

Day 2
- **miRNA analysis:**
  - GMS demo: mapping to smallRNA library
  - GGA hands-on: differential miRNA expression
- **RNA analysis:**
  - GMS demo: spliced mapping to genome mapping to transcriptome
  - GGA hands-on: differential expression analysis
  - biological classification and pathway analysis of differentially expressed genes
  - assessment and visualization of alternative exon/transcript usage
- **ChIP-Seq analysis:**
  - GGA hands-on: peak detection and classification
  - TF binding site analysis in ChIP peaks
  - de novo definition of common sequence motifs in ChIP data
  - next-neighbor analysis and regulatory target prediction for ChIP regions
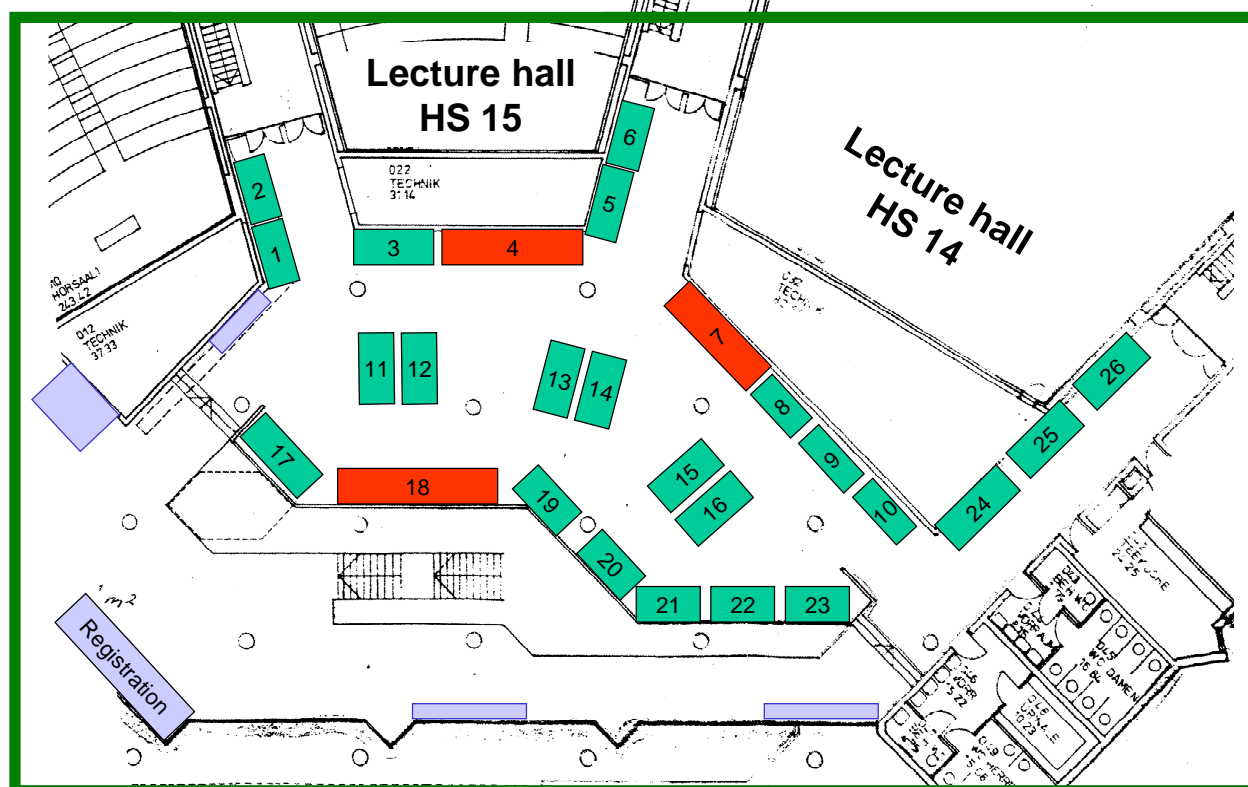 - correlation of several data sets

Industrial Exhibition

40 companies participate in the industrial exhibition held during the qPCR Symposium March 18th – 20th in the foyer of the central lecture hall complex (green frame) and in two exhibition side rooms S1 and S2 (blue frame).

Booth	Company	Booth	Company
1	Kapa Biosystems / Peplab	18	Life Technologies
2	Solis Biodyne	19	Biolegio
3	Bioline / 4titude	20	Fluidigm
4	Bio-Rad	21	Eppendorf
5	New England Biolabs / Bioke	22	Eurogentec
6	Eurofins MWG Operon	23	Quanta Biosciences
7	Roche Applied Science	24	Integrated DNA Techn.
8	Qiagen	25	Illumina
9	Nanostring	26	Gilson
10	Agilent Technologies	27	TATAA & MultiD
11	Metabion	28	Analytik Jena
12	Clontech / TaKaRa	29	Biogazelle
13	Biozym / Cyclertest	30	Mikrogen Diagnostik
14	BioSistemika	31	Hamilton Robotics
15	Biosearch Technologies	32	Primerdesign
16	Exiqon	33	Wafergen
17	TIB Molbiol	34	RainDance Technologies

S2

S1

Kitchen

Rest Rooms

Rest Rooms

Main Exhibition Area in Foyer
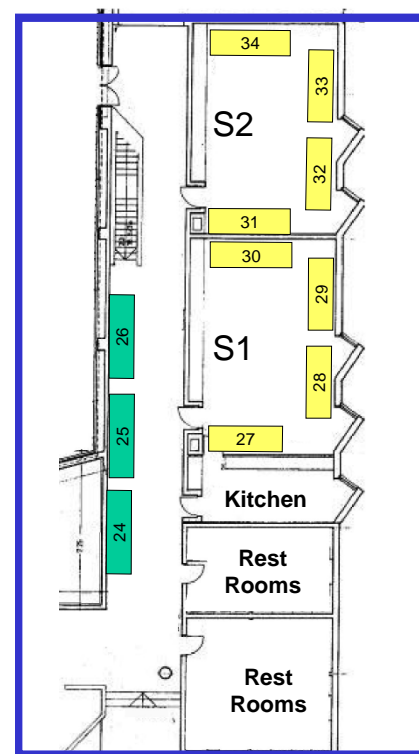
- Registration & Catering
- Booth - Foyer
- Booth - S1 & S2

Lecture hall HS 15

Lecture hall HS 14

Registration

Abstracts – Oral Presentations

Session - MD 1: Molecular Diagnostics 1

Time: **Monday, 18/Mar/2013: 10:15am - 1:15pm**
Session Chair: **Michael W Pfaffl**, TUM, Germany
Session Chair: **Mikael Kubista**, TATAA Biocenter, Sweden

Location: **Lecture hall 14**
650 participants, TUM Weihenstephan

Presentations

Near-Oncology Patient (and Near-Oncology Health Care Provider) Molecular Testing on the GeneXpert

Russell Higuchi

Cepheid Fellow R&D, Cepheid, Sunnyvale, CA, USA

In this talk, I will describe our efforts to provide these testing features for cancer diagnostics. In particular, I will cover current and upcoming approved tests for BCR-ABL translocation monitoring in CML patients and for the non-invasive detection of bladder cancer occurrence and recurrence. I will also describe our pilot efforts to develop rapid, bench-top assays to aid the histo-pathologist assess the molecular status of tumors from formalin-fixed, paraffin-embedded biopsy sections. We believe that by bringing such testing directly into the hands of the histo-pathologist, faster and better patient treatment decisions can be made.

Features of the Cepheid GeneXpert molecular diagnostics system include:
1) Full integration of real-time, six-color PCR with an automated sample-prep capable of efficient extraction of large, biologically relevant sample amounts. Samples include sputum, blood, urine and stool.
2) A disposable cartridge format that allows rapid "sample-in, answer-out" with sample and PCR-product containment.
3) A modular, "host" system for the cartridges that provides for process control and multiplex, real-time PCR product detection. Like for computer servers in a rack, the number of modules is expandable as needed – from a single module to 80. Systems with 48 and 80 modules provide for walk-away, automated queuing and loading of sample-filled cartridges.
These features have helped bring the benefits of molecular testing for infectious disease to sites as diverse as the reference lab, the hospital lab and clinics in remote parts of the developing world.

High throughput mRNA and protein expression profiling by qPCR

Mikael Kubista[1,2], **Robert Sjöback**[1], **Jens Björkman**[1], **David Svec**[1], **Anders Stahlberg**[1,4], **Vendula Rusnakova**[2], **Miroslava Anderova**[3]

[1]TATAA Biocenter, Sweden; [2]Institute of Biotechnology, Czech Academy of Sciences; [3]Institute of Experimental Medicine, Czech Academy of Sciences; [4]Cancer center, University of Gothenburg

qPCR is developing into the most powerful platform to profile samples extracting the biologically relevant information. Major breakthrough in recent years is the development of robust preamplification methods, which allow the profiling of large number of markers starting with minute sample amounts, including single cells. In first part of my talk I will describe high throughput single cell profiling of astrocytes collected over-time from mouse brains after induced trauma. The profiling reveals how the brain responds to the injury by activating the astrocytes reacting to the trauma and inducing repair. This multiway study with expression of many genes, measured in large number of cells, over time is analyzed with powerful multivariate tools in GenEx from MultiD to identify clusters of coregulated genes, which biological functions and expression pathways are then mapped using the Ingenuity iReport. In the second part of my talk I will present high throughput protein expression data measured with qPCR using Olink Proximity extension Assays combined with preamplification.

Sequence, Shape, Function: Synthetic Biology by DNA

Hendrik Dietz

Technische Universität München, Germany

Many processes in biology rely fundamentally on the relative position and orientation of interacting molecules. It is notoriously difficult to observe, let alone control, the position and orientation of molecules because of their small size and the constant thermal fluctuations that they experience in solution. Molecular self-assembly with DNA provides a route for placing molecules and constraining their fluctuations in user-defined ways, thereby opening attractive avenues for scientific and technological exploration. In the three parts of my talk I will provide evidence for this statement:

(1) Positional Control - The field has faced scepticism regarding its viability for creating objects with sufficient order and homogeneity to confer utility. I will present a high-resolution 3D structure of a discrete DNA based object that is twice the size of a prokaryotic ribosome [1]. The structure confirms structural order in synthetic DNA objects that is comparable to those found in proteins and supports a perspective in which chemical motifs may be arranged with precise structural specifications through an iterative strategy of DNA-templated design and 3D structural feedback. By using chemical groups attached to DNA strands or even reactive motifs formed by DNA itself, this strategy offers an attractive route to achieving complex functionalities known today only from natural nanomachines.

(2) Practical Assembly - In recent years, design strategies for encoding complex target shapes in DNA sequences have flourished, but the practical assembly of desired objects has often been quite difficult. I will show that, at constant temperature, hundreds of DNA strands can cooperatively fold a long template DNA strand within minutes into complex nanoscale objects [2]. Folding at optimized constant temperatures enabled the rapid production of DNA objects with yields that approached 100%, thereby opening attractive prospects for converting DNA-based self-assembly into a real-world manufacturing technique.

(3) Application - Finally, I will present synthetic lipid membrane channels that we created from self-assembled DNA nanostructures [3]. In single-channel electrophysiological measurements, we found similarities to the response of natural ion channels, such as conductances on the order of 1 nanosiemens and channel gating. More pronounced gating was seen for mutations in which a single DNA strand of the stem protruded into the channel. Single-molecule translocation experiments show that the synthetic channels can be used to discriminate single DNA molecules.

[1] Bai et al, PNAS, Dec 4 2012;

[2] Sobczak et al, Science, Dec 14, 2012;

[3] Langecker et al, Science, Nov 16 2012

The New LightCycler® 96 System: It Is So Easy To Be A Lab Hero

Ralf Peter Mauritz

Roche Diagnostics GmbH, Germany

The new LightCycler® 96 system is a benchtop instrument that can handle up to 96 samples using standard 96 multi well plates or 8-tube strips. A new optical module is developed that is based on 2x 96 glass fibres with 4 excitation and 4 emission filters. A white LED is used as light source and a CCD camera as detection unit. This setup supports the most common PCR formats used in the market like SYBR Green I and Hydrolysis Probes and thus enables the user to perform all common PCR applications.

The instrument can be operated by a PC or in a stand-alone configuration via a touch screen. Experiments can be handled either by USB stick, by LAN or by a connected PC / laptop. The software for run and analysis offers new workflows and features compared to other state-of-the-art PCR cycler software. While online monitoring of PC reactions is possible from any PC within a local network also Email notification including executed experiment data is supported by the new LightCycler® 96 System Software. All major analysis modules like Abs Quant, Rel Quant, Tm Calling, Endpoint Genotyping, Qualitative Detection and High Resolution Melting are provided. Full support of the new PCR standard guidelines is given by providing a RDML (Real-Time PCR Data Markup Language) compatible experiment file following the MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) rules.

Thus, the new LightCycler® 96 Real-Time PCR System delivers speed, reliability and accuracy combined with a new software solution that makes it easy to be a lab hero.

LightCycler® is a trademark of Roche.

A Molecular Assay With Laser-heated Nanoparticles

Lars Ullerich

GNA Biosolutions GmbH, Germany

GNA Biosolutions, a nanotechnology start-up based in Munich, Germany, develops assays and instruments for ultra-fast DNA detection, based on GNA's optothermal NANOSTOVE technology.

Instead of fluorophores, GNA employs nanoparticles, functionalized with probe DNA sequences. The nanoparticles are heated locally by short laser pulses, leading to optically measurable de-hybridization events between nanoparticle probe and target sequences.

We have shown DNA-based pathogen detection from sample to results in less than 20 minutes with our current diagnostic test assays. GNA's current pipeline encompasses a set of DNA assays for diverse pathogens. Conventional 'fast' methods for pathogen detection typically take at least 90 minutes, and most often rely on the infrastructure of central laboratories. NANOSTOVE technology will enable much faster, point of care detection of pathogens and antibiotic resistances.

Founders and managing directors of GNA are Dr. Joachim Stehr (head of research), Dr. Federico Bürsgens (finances and hardware development), and Dr. Lars Ullerich (biotechnology and business development). GNA Biosolutions is a spin-off from the Institute for Photonics and Optoelectronics at the University of Munich, and was founded in 2010.

Going to the limits of Multiplex Real-time PCR

Olfert Landt[1], Ulrich Lass[1], Matthias Ballhause[1], Johannes Kusters[2], Pranav Patel[3]

[1]Tib Molbiol Syntheselabor GmbH, Berlin, Germany; [2]Medical Microbiology, University Medical Center Utrecht, The Netherlands; [3]Robert-Koch-Institut, Berlin, Germany

Common opinion is that single target PCR is more sensitive than multiplex PCR, and indeed there are more putative interactions when more primers are present in the reaction. However, multiplex PCR reduces costs and handling efforts and is particular interesting for screening purposes. Although there are many multiplex assays published, we noted that they are rarely used in clinical routine diagnostics. We present examples for diagnostic use hexaplex TaqMan assays on a LightCycler 480 II system - the current limit is the number of available dye channels - for detection of bacteria, parasites or different ESBL gene targets, and evaluation data for two gastrointestinal assays as well as some data obtained with the LightCycler Nano instrument, running even more than six assays. Since the typical feedback from laboratories was the desire to exclude single assays, or to exchange them for assays with a different specification, we developed a novel concept of modular assays, which can be combined according to the respective clinical requirement. Last not least we will present first results from multiplex Recombinase-Polymerase-Amplification (RPA) assays designed for the detection of different Coronaviruses, and discuss the exciting opportunities for an ultrafast point-of-care screening for infections.

Session - NGS 1:   Next Generation Sequencing 1

Time: **Monday, 18/Mar/2013: 2:00pm - 6:00pm**
Session Chair: **Vladimir Benes**, EMBL GeneCore, Germany
Session Chair: **Alexander Schramm**, University Hospital Essen, Germany

Location: **Lecture hall 14**
650 participants, TUM Weihenstephan

Presentations

RNA-Seq: opportunities, limitations and applications in cancer research

Alexander Schramm[1], Marcel Martin[2], Johannes H. Schulte[1], Johannes Köster[2], Pieter Mestdagh[3], Jo Vandesompele[3], Sven Rahmann[2,4]

[1]University Hospital Essen, Pediatric Oncology, Germany; [2]TU Dortmund, Dept. of Computer Science, LS11, Germany; [3]Center for Medical Genetics Ghent, Belgium; [4]University Hospital Essen, Genome Informatics, Germany

Next generation RNA sequencing allows for the detection of aberrantly expressed transcripts associated with cancer biology and outcome, thus enabling the identification of biomarkers and therapy targets on transcriptome level. Additionally, using both high-throughput sequencing and quantitative real-time PCR, the transcriptome can be analyzed in complementary ways. In two pilot studies, we could demonstrate that deep sequencing of both mRNA and miRNA allows for pathway and pattern identification using the embryonal tumor, neuroblastoma, as a model system. We have established the necessary bioinformatics pipeline, including software tools, and key methodological steps in the process, such as adapter removal, read mapping, normalization, and multiple testing issues for biomarker identification. The computational pipeline for obtaining a ranked list of differentially expressed miRNAs from the raw sequence reads was also standardised and methods for comparison of NGS and qPCR data have been implemented. Fundamental challenges involving estimation of expression values from short RNA reads as well as mapping of short reads will be discussed.

Advancements in Ion Torrent RNA Sequencing: More and Less

Richard Fekete, Kelli Bramlett, Yongming Sun, Jeff Schageman, Luming Qu, Ross Hershorn, Charmaine San Jose Hinahon, Brian Sanderson, Angie Cheng, Bob Setterquist

Life Technologies, United States of America

RNA-Seq technology has become widely utilized as a tool to understand the transcriptome of a given experimental system. This method utilizes next generation sequencing platforms to sequence a cDNA library in order to gain information about the RNA content and transcriptional status of a sample of interest. Profiling the transcriptome of a system in this way has become an invaluable tool in many genomic studies.

More information is now available through the use of the newly launched Proton™ system utilizing the same simplified chemistry first introduced with the Personal Genome Machine (PGM™). The increased sequencing depth of the Proton™ instrument and the Ion PI™ chip now lends this simplified sequencing technology to true whole transcriptome evaluation including sequencing analysis of polyadenylated RNAs, long non-coding RNAs, and non-adenylated transcripts. We describe the differential gene expression profiling of two well-studied RNAs, Universal Human Reference RNA (UHRR) and Human Brain Reference RNA (HBRR), with the External RNA Control Consortium transcripts (ERCCs) spiked into the RNAs. This study demonstrating over 50M mapped reads from each Proton™ transcriptome sequencing run, comparable gene expression profiles to an orthologous technology, and good sensititivity and dynamic range as shown with the ERCC controls, solidifies the new Proton™ sequencing system as a optimal platform for complex transcriptome analysis.

Less information is more appropriate when only a handful of the genes present are necessary to make a decision such as a clinically relevant diagnosis. We have demonstrated new technology that allows the expression analysis from a panel of selected genes by sequencing directed amplicons using an AmpliSeq™ approach with Ion Torrent semiconductor sequencing. This approach offers many advantages over microarray or qPCR such as faster turnaround and data analysis, sample multiplexing, lower RNA inputs, and ability to use degraded or FFPE-derived samples. In addition, the technique simultaneously provides quantitative gene expression information and gene sequence at the single nucleotide level. We have developed two ready-to-use gene panels targeting cancer and apoptotic pathways. Gene specific amplicons are prepared for sequencing using the AmpliSeq™ technology and sequenced using Ion Torrent sequencing technology. We demonstrate that the technique produces results that are technically reproducible, quantitative, and have excellent correlation with qPCR using TaqMan® gene expression assays. Employing barcodes, we have also multiplexed AmpliSeq™ RNA libraries thereby increasing the cost-effectiveness of the tool for higher throughput laboratory settings.

The Potential for Next Generation Sequencing in Forensics

Nicola Oldroyd

R&D, Illumina, United Kingdom

Genetic analysis continues to transform forensic investigations, with each technology advance enabling scientists to extract more and more information from forensic samples. The latest tool is next-generation sequencing (NGS), which has significantly enhanced biological research since its introduction in the mid-2000s.

NGS is just beginning to be used in the Forensic Genomics laboratory, with early studies in standard tandem repeat (STR) typing, mitochondrial DNA analysis, and dense panels of single nucleotide polymorphisms (SNPs) offering a tantalizing view of its advantages in revealing Forensic DNA evidence from even the smallest, most fragile, and highly mixed samples.

Advances in NGS library preparation – the devil is in the detail

Bianka Baying, Bettina Haase, Jonathon Blake, Dinko Pavlinic, Jürgen Zimmermann, Vladimir Benes

EMBL-GeneCore, Meyerhofstr. 1, 69117 Heidelberg

Next generation sequencing (NGS) offers an unprecedented opportunity to characterize comprehensively components of the entire cellular nucleic acids content, including its modifications such as cytosine methylation, for example, and RNA or DNA sequences bound by their cognate proteins. However, with the exception of genomic DNA, whose content in the cell is more or less constant (apart from amplifications associated with various pathological conditions), nucleic acid levels in the cell are highly variable due to cell state dynamics. The complexity is furthermore compound by the variations in source, amount and quality of nucleic acid under investigation.

So far there is no NGS system that enables us to explore individual cellular nucleic acid components without a 'pre-processing' step, i.e. preparation of sequencing library. Ideally, each library should reflect sample complexity without missing anything. However, variable abundance of investigated nucleic acid molecules, their uneven base composition, inefficiencies of *in vitro* reactions or unrecognized sequence preferences of used enzymes make this goal hard to achieve. Even the way RNA molecules are fragmented impacts on the representation of particular transcripts in the obtained data. Introduction of magnetic beads, developments in enzymology and miniaturization of library preparation protocols have considerably improved our capability to prepare good sequencing libraries, resulting in certain applications even in the omission of a PCR step. The market place continues bringing out new applications and protocols to deal with issues seen in NGS library preparation. We have approached these critically when adopting new products in our workflows.

The NeXT generation Variant annotation Tracker: a one stop cloud solution to exome sequencing data analysis

Bram De Wilde, Tom Sante, Jasper Anckaert, Jan Hellemans, Frank Speleman, Björn Menten, Jo Vandesompele

Center for Medical Genetics, Ghent University, De Pintelaan 185, 9000 Gent, Belgium

Background - As genetic variation data is being generated at an unprecedented scale, assessment of functional consequences of the variants in a given patient or patient cohort is a challenging task, both from a computational as from a data management perspective. It is expected that in this new era of personalised genomics, a clinical sample may need to be re-annotated repeatedly as new annotation information on the genome becomes available and new insights on variant interpretation accumulate. More so, the advent of individualised, genomics directed therapeutical strategies will require patients tumours to be genetically profiled at multiple levels within the diagnosis to treatment timeframe, thus requiring the fast and qualitative analysis of huge datasets. While various initiatives emerge to collect the overwhelming amount of genomic variants currently generated, a central system to manage and store the annotation of genomic variants on a sample by sample basis is still missing.

Results - Here we present our efforts to create a one stop solution to next generation sequencing data analysis. The 'NeXT-generation Variant Annotation Tracker' is a front-end to a highly scalable cloud based analysis platform. Combining this web based front-end with an object oriented shardable database and a fully distributed analysis pipeline allows us to scale this application to virtually any size required. A "plug in" style organisation of the variant annotation pipelines makes updating and extending variant annotation easy. The cloud based nature of this platform addresses both the scalability and data management issues encountered when working with huge next generation sequencing datasets.

Visualisation of variant annotation at the individual samples but also on the population level can easily be achieved through a map reduce framework allowing us to grasp the genomic variation at both the gene or biological pathway level.

Currently variant annotation and effect prediction is done using the Ensembl API (1), the polyphen2 algorithm (2) and genesplicer (3). All tools are fully compatible with the emerging standard formats in next generation sequencing data analysis, including the VCF version 4.1 from the 1000 genomes consortium (4,5).

1. Mclaren W et al. Deriving the consequences of genomic variants with the Ensembl API and SNP Effect Predictor. Bioinformatics 2010 26(16): 2069–70.
2. Adzhubei IA et al. A method and server for predicting damaging missense mutations. Nat Meth. 2010 Apr 1;7(4):248–9.
3. Pertea M et al. GeneSplicer: a new computational method for splice site prediction. Nucleic Acids Res. 2001 Mar 1;29(5):1185–90.
4. Danecek P et al. The variant call format and VCFtools. Bioinformatics. 2011 Jul 15;27(15):2156–8.
5. Durbin RM et al. A map of human genome variation from population-scale sequencing. Nature. 2010 Oct 28;467(7319):1061–73.

Transforming NGS for clinical research and diagnostics

Erik Söderbäck

Market Development Manager, Qiagen, Germany

Next-generation sequencing (NGS) is expected to increase its transformational impact on healthcare and diagnostics; however, current offerings do not meet the demands of the clinical environment. QIAGEN's highly automated NGS workflow addresses these demands by offering a streamlined and standardized ecosystem built on components that include a fully automated nucleic acid purification and library preparation process, based on proven QIAcube technology; new GeneRead DNAseq Target Enrichment Gene Panels designed using our proprietary GeneGlobe collection of more than 60,000, fully annotated, molecular assays; a new instrument for automated preparation of sequencing template; and GeneReader, a new benchtop sequencer that embraces diagnostic workflow features and offers a high level of flexibility, scalability, and efficiency. A dedicated QIAGEN software solution for powerful, yet user-friendly and automated result analysis, completes the new portfolio. The GeneReader features a turntable with up to 20 flow cells to process samples independently, and in parallel, with great efficiency. QIAGEN GeneGlobe provides easy access to pre-validated gene panels, as well as to custom gene content. The system is designed to offer random access and continuous load processing — key benefits in the clinical research and diagnostics segments, which are faced with higher sample throughput requirements.

New developments in NGS target enrichment

Götz Frommer

Sales Manager Genomics Germany, Agilent Technologies Diagnostics and Genomics Group, Germany

As Next Generation Sequencing (NGS) becomes more affordable and transitions to use in clinical research for mutation detection, it is essential for researchers to have an efficient end-to-end workflow, including target enrichment panel design, sample preparation, sample QC, sequencing, and data analysis. Agilent's SureSelect and HaloPlex technology for target enrichment, SureDesign application for panel design, and SureCall application for data analysis combine with today's desktop sequencers to make this workflow a reality.

SureSelect and HaloPlex are target enrichment systems ideally suited to deep sequencing of relatively small panels of genes up to whole exomes. The Agilent Target Enrichment System enables fast, simple, and efficient analysis of genomic regions of interest for a large number of samples, covering thousands of exons per sample and is compatibel with different desktop sequencing and high-throughput platforms.

SureDesign is a web-based design application for designing custom panels. With this tool, researchers can quickly generate high-coverage, high-efficiency designs for targeted re-sequencing.

SureCall is an easy-to-use desktop application combining best in class open source algorithms for end-to-end NGS data analysis from alignment to categorization of mutations.

Recent developments in sample QC with the 2100 Bioanalyzer and the 2200 Tapestation, the automation of the workflow based on the Bravo Liquid Handling System and various applications, such as RNA-Seq and Methyl-Seq will be presented.

Session - dPCR: digital PCR

Time: **Tuesday, 19/Mar/2013: 8:30am - 12:30pm**
Session Chair: **Jim Francis Huggett**, LGC, United Kingdom
Session Chair: **Jo Vandesompele**, Ghent University / Biogazelle, Belgium

Location: **Lecture hall 14**
650 participants, TUM Weihenstephan

Presentations

An introduction to digital PCR

Jim Francis Huggett

LGC, United Kingdom

Digital PCR (dPCR) reflects the latest incarnation of the polymerase chain reaction that converts its output into a binary signal. This is achieved by performing limiting dilution of the sample into a large number of reactions (partitions) so that a proportion contain no template. The proportion of positive and negative partitions are measured and used to estimate the concentration of target molecules present. Unlike qPCR, dPCR does not require a standard curve for accurate and sensitive measurement and potentially offers more precise quantification as well as improved fold change measurement and detection of minority target information. This presentation will discuss the benefits of this approach as well as some of the disadvantages, highlight the MIQE considerations that apply to dPCR and offer a prediction of how dPCR may impact on molecular measurement in the future.

Performance Of A Next Generation Fixed Microwell Platform For Digital PCR

David N. Keys

Life Technologies, United States of America

Digital PCR enables specific nucleic acid sequences to be quantified with high accurately, precision, and sensitivity. A large number of novel applications are enabled by this new approach, including reference free absolute quantification and high sensitivity rare-allele quantification. With the advent of novel nanofluidic PCR technologies, running the thousands of reactions required for digital PCR has become feasible. To this end, Life Technologies has developed the QuantStudio™ 3D Digital PCR System, a new silicon chip-based digital PCR platform that can be rapidly loaded with little to no dead volume. At the heart of the nanofluidic system is a chip that enables samples to be partitioned into 20,000 fixed volume reactions. The system utilizes a three step workflow optimized for simplicity, minimal hands-on time, minimal opportunity for cross contamination, and minimal sample loss. We will present results on digital PCR performance which can be achieved with this system, with emphasis on absolute quantification and rare-allele quantification in cancer derived cell lines and tumor samples.

Quantitative Detection of Cancer Biomarkers in Picoliter Droplets.

Valerie Taly[1], Deniz Pekin[1,3], Corinne Normand[1], Zakaria El Harrak[1], Thevy Hor[1], Li Xinyu[2], Ivan Atochin[2], Steve Kotsopoulos[2], Delphine Le Corre[1], Leonor Benhaim[1], J. Brian Hutchison[2], Darren R. Link[2], Helene Blons[1], Philippe Nizard[1], Pierre Laurent-Puig[1]

[1]Université Paris Descartes; INSERM UMR-S775; Centre Universitaire des Saints-Pères, 45 rue des Saints-Pères, 75270 Paris Cedex 06, France;[2]RainDance Technologies, Lexington, MA 02421, Massachusetts, USA; [3]Université de Strasbourg; CNRS UMR 7006, 8 allée Gaspard Monge, BP 70028, F-67083 Strasbourg Cedex, France

Gene alterations within tumoral DNA can be used as highly specific biomarkers to distinguish cancer cells. These DNA biomarkers are especially important for the diagnosis, prognosis, treatment and follow-up of patients. Target-oriented treatments have dramatically improved prognosis of patients with cancer and new biomarkers of resistance or sensitivity to such treatment have been highlighted (1). These drugs are now limited to subgroups of patients including, for example, patients bearing *KRAS* wild-type colorectal adenocarcinomas for anti-EGFR antibodies based treatments. However it has also been recently demonstrated that resistance occurs through the selection of resistant subclones. In case of resistance of colorectal cancer to cetuximab and panitunimab subclones, such subclones bear *KRAS* mutations.

Plain use of gene alterations as biomarkers in clinical oncology requires a highly sensitive, and ideally quantitative, strategy that allow the detection of the tumour specific modifications in a background of non-mutated DNA from normal cells (2). Procedures classically used in clinics cannot detect less than ~1% mutant genes in a background of non-mutated DNA from normal cells. Among the challenge to be assessed in clinical oncology, the ability to detect with a high sensitivity and quantify mutations in complex samples (including tumors or plasma) will allow, within other, to detect subclones in order to predict resistance and build therapeutic strategies to overcome potential resistance as well as to perform follow up of the efficiency of treatment by measuring disappearance of the targeted clones. Moreover, such procedure will permit to use non-invasive strategies that could greatly facilitate patient follow up (3).

We first developed a procedure based on using droplet-based microfluidics to perform digital PCR in millions of picoliter droplets (4). By segregating individual target DNA-molecules in billions of aqueous droplets acting as independent microreactors, this procedure allows extremely precise, sensitive and fast quantification of mutated genes to be carried out. The procedure has then been extended the detection of multiple mutations in parallel (5). Finally, to demonstrate the pertinence of our procedures to overcome the clinical oncology challenges, two studies have been built. When the first one addresses our ability to detect rare subclones in colorectal tumors and to understand the impact of these subclones on responses and survival of the patients treated by anti-EGFR therapies, the second one aims at demonstrated the possibility to detect *KRAS*mutations directly in plasma of patients with advanced colorectal cancers.

1. Sawyers CL, Nature 2008, 452(7187), 548-552; 2. Diehl F, et al., Curr Opin Oncol 2007, 19(1), 36-42; 3. Taly V, et al., Trends Mol Med 2012, 18(7), 405-416. 4. Pekin D, et al., Lab Chip 2011, 11(13), 2156-2166. 5. Zhong Q. et al., Lab Chip 2011, 11(13), 2167-2174.

Enhanced Resolution of Copy Number Variants in Domestic Animal Species

Jennifer Meadows

Uppsala University, Sweden

Modern domestic animal species represent a special combination of natural selection to their new environment (e.g. the farm) and human mediated selection for visible traits of interest. This amalgamation created not only an excellent model organism for the genetic dissection of phenotypic traits, but also provided an invaluable resource in which evolution in action could be studied. We can interrogate the genomes of domestic animals as we would a manufactured genetic screen; only in this setting mutations are spontaneous, primarily non-deleterious and potentially cumulative. Structural mutations (duplications, deletions, inversions or copy number expansions) have been shown to underlie more than a dozen of the phenotypic traits investigated by our comparative genetics group. I will provide case studies to illustrate the past methodologies used by us to quantify these events, and will demonstrate how this resolution can be improved with the application of digital PCR technology. I will also show how in one instance, quantification gains aided in the interpretation of copy number transmission and regulatory action.

RainDrop Digital PCR: Single Molecule Counting With A Droplet Digital PCR Platform

Adam Corner

RainDance Technologies, United States of America

RainDance Technologies' RainDrop™ digital PCR system is a highly sensitive tool for precise quantification of nucleic acids using standard probe-based qPCR reagents. The RainDrop offers unique analytical advantages, providing exceptionally high sensitivity without the need for standard curves, and having the capability to multiplex assays, using a wide dynamic range of input DNA in a contamination-free and simple workflow. In this presentation you will learn the basic principles of digital PCR as well as see details of the RainDrop dPCR system and examples of its use in various applications.

Use Of Digital PCR For Improved Copy Number Quantification

Ariane De Ganck[1], Annelies Dheedene[2], Björn Menten[2], Jan Hellemans[1], Jo Vandesompele[1]

[1]Biogazelle, Zwijnaarde, Belgium; [2]center for medical genetics, Ghent University, Ghent, Belgium

Although the expertise of Biogazelle is historically based on quantitative PCR, we welcome the opportunity to be able to offer digital droplet PCR to our customers for a number of cases were qPCR could not fulfill their needs.

Here we present a pilot study for the genetic characterization of cell banks used for the production of recombinant therapeutic proteins. Determination of transgene copy number and genetic stability are important parameters for recombinant protein production. Using ddPCR, a markedly higher accuracy and precision in terms of copy number determination was obtained. Moreover fewer replicate reactions are necessary compared to qPCR. This study paves the way for ddPCR to become the gold standard for transgene copy number determination.

A second case study we will present is part of an internal R&D project in collaboration with Ghent University set up to discover the ddPCR boundaries for noninvasive prenatal diagnosis (NIPD) of chromosomal aneuploidies. The discovery of cell-free fetal nucleic acids in maternal plasma has opened up new possibilities in this context. Our current results support the use of ddPCR to determine chromosomal aneuploidy in post-natal samples. More efforts in terms of assay design, multiplexing strategy and data normalization are ongoing to improve accuracy and precision to enable the use of ddPCR for NIPD.

The results obtained in these two case studies confirm the unique potential of ddPCR for copy number analysis in both clinical and research settings.

Droplet Digital PCR For Free Fetal DNA Analysis: Statistical Modelling And Evaluation For Non-Invasive Prenatal Diagnosis Of An X-linked Deletion.

Emmanuel Debrand[1], Michael Samuels[2], Sarah Clinton[1], Stephanie Allen[1]

[1]Birmingham Women's Hospital, United Kingdom; [2]RainDance Technologies

The discovery of cell-free fetal DNA (cffDNA) in maternal plasma has allowed the development of non-invasive prenatal diagnostic (NIPD). However, as cffDNA only represents a proportion (2-20%) of total cell-free DNA, it has been difficult to determine whether a fetus has inherited a maternal mutation using conventional techniques.

Single molecule counting techniques, such as digital PCR (dPCR), have been investigated for NIPD of such mutations as this requires precise quantification of mutant and wild type alleles and determination of whether there is an allelic imbalance. The direction and amplitude of the imbalance are directly influenced by the fetal genotype and the fraction of cffDNA present. Such a Relative Mutation Dosage (RMD) strategy has been used to infer fetal genotype from imbalances measured by dPCR in various clinical situations, although with a sensitivity which may not be suitable for clinical use. Existing studies have used first generation dPCR systems based on nanofluidic arrays, which provide a limited number of individual PCR chambers and may lack the precision and sensitivity required. In contrast, the more recent droplet digital PCR (ddPCR) approach appears promising in terms of number of partitions and cost but has not yet been investigated in the context of NIPD.

To address this, we adapted Power calculation methods based on existing statistical models of the variance associated with dPCR measurements and used them to predict the effect of number of partitions (chambers or droplets), total quantity of DNA available and fetal fraction, on the ability to discriminate between two fetal genotypes. Data will be presented that suggests: (i) that both number of partitions and sample quality are crucial determinants of the sensitivity achieved by dPCR in measuring allelic imbalances; (ii) that the larger number of partitions provided by ddPCR systems should in turn lead to a much improved precision and sensitivity.

Deletions and duplications within the X-linked dystrophin gene are a major cause of Duchenne and Becker Muscular Dystrophy (DMD/BMD). We therefore developed a dPCR assay design capable of measuring imbalances due to the presence or absence of a DMD deletion in a male fetus. We will show that determination of the male fetal fraction is achievable on two different ddPCR platforms (RainDrop and BioRad QX100), with a sensitivity and precision superior to that achieved on an array-based platform. Furthermore, a multiplexed assay was developed on the RainDrop platform that enables simultaneous determination of both male fraction and DMD balance (relative to X chromosome) in a single test. Optimisation, sensitivity and performance data of the DMD-specific assay using artificial mixtures of various genotype combinations relevant to clinical practice will also be presented and discussed in particular with respect to the number of droplets generated by each system and the predictions of our Power calculation tools.

Session NGS 2: Next Generation Sequencing 2

Time: **Tuesday, 19/Mar/2013: 8:30am - 12:30pm**
Session Chair: **Robert P. Loewe**, GeneWake GmbH, Germany
Session Chair: **Michael W Pfaffl**, TUM, Germany

Location: **Lecture hall 15**
350 participants, TUM Weihenstephan

Presentations

The Road to Genomic Medicine is Paved with Data and Information

John Quackenbush

Dana-Farber Cancer Institute, United States of America

Since the introduction of second-generation DNS sequencing technologies in 2007, the cost of genome sequencing has been consistently by 33% per quarter, with the $1000 genome arriving in 2012 and the $100 genome not far off. As DNA sequencing increasingly becomes a commodity, biomedical research is rapidly evolving from a purely laboratory science to an information science in which the winners in the race to cure disease are likely to be those best able to collect, manage, analyze, and interpret data. Here I will provide an overview of the approach we have been developing to deal with the challenge of personal genomic data, including integrative approaches to data analysis and the creation of data portals focused on addressing the most common use cases presented by different user constituencies. By effectively collecting genomic and clinical data and linking information available in the public domain, we have made significant advances in uncovering the cellular networks and pathways that underlie human disease and building predictive models of those networks that may help to direct therapies.

Sequencing the "Unsequenceable" Single-Molecule, Real-Time (SMRT™) DNA Sequencing: Technology Overview and Recent Applications

Ralph Vogelsang

Pacific Biosciences, United States of America

Understanding the dynamics of biological processes is fundamental to understanding life itself. At Pacific Biosciences, we are developing applications to observe individual biomolecules at work in real time. The first is monitoring DNA synthesis by single DNA polymerase molecules, allowing the speed, processivity, and efficiency of the enzyme to be exploited for new capabilities in DNA sequencing. The power of this new sequencing technology - characterized by long readlengths and fast run times - is highlighted through examples from diverse applications, such as finishing genomes (de novo hybrid assembly), targeted resequencing of difficult to sequence regions like GC- or AT-rich, characterizing transcript and gene fusion diversity, rapid pathogen sequencing, and the direct detection of epigenetic base modifications by making use of the kinetic information collected during DNA-sequencing.

Droplet Digital PCR and Next Gen Sequencing: Digital Biology in High Definition

Svilen Tzonev

Bio-Rad, United States of America

Advances in digital PCR and Next Gen Sequencing technologies continue to expand the use of digital approaches in Biology. Both techniques produce large amounts of digital, high definition data and are natural complements to each other. This presentation will discuss droplet digital PCR technologies and applications developed by Bio-Rad and its customers. Some examples include absolute DNA quantitation, copy number analysis, rare mutation and rare species detection, gene expression down to the single cell. Emerging applications include viral load detection, GMO load, carrier status, phasing enzymatic activity and many others. We will also discuss application of ddPCR for NGS library quantitation and quality control and novel approaches for sample prep in droplets for sequencing.

The WaferGen's SmartChip System: "Cycling in the world of high- throughput qPCR and target enrichment"

Stefaan Derveaux

WaferGen Biosystems Europe S.à r.l., Luxembourg

The SmartChip Real-Time PCR System is a next-generation quantitative PCR platform that combines the high throughput nature of microarrays with the sensitivity, precision and dynamic range of quantitative real-time PCR, and a high level of flexibility. Whether in discovery or validation environments, assay/sample formats can be easily determined for maximum efficiency and cost savings. At the hearth of the system is the SmartChip with 72x72 nanoscale-wells, each with an effective reaction-volume of 100 nL. This configuration allows completion of 5184 quantitative real-time PCR reactions in just 2 hours. Using the pre-spotted panels, 1 sample can be analyzed for thousands of targets at once, but the MyDesign approach also allows to add assays and samples to a chip -all in the convenience of your own lab- and to analyse just a few targets in several hundreds of samples in one qPCR run. In this talk we present multiple gene expression and genotyping studies to demonstrate the flexibility of the platform. One of the main challenges in conducting accurate and efficient targeted re-sequencing using next-gen methods is the target enrichment step. WaferGen recently established a PCR-based target-amplification workflow for next generation sequencing using the same high-density SmartChip technology, allowing one to enrich hundreds to thousands of target regions by amplifying each target in an individual PCR reaction thereby avoiding multiplexing interference. We will show preliminary results of the first studies that were performed in collaboration with our early-access users, demonstrating the overall quality and coverage uniformity that was obtained. We also highlight new products that were developed to this end.

The distribution of small RNAs in milk and their functional relation to mammary gland physiology

Michael W Pfaffl[1], Benedikt Kirchner[1], Alexander Hahn[2], Vladimir Benes[3]

[1]Physiology Weihenstephan, Technical University Munich, Germany; [2]Genomatix Software GmbH, Munich, Germany; [3]EMBL Genomics Core Facility (GeneCore), Heidelberg, Germany

Small RNAs, in particular microRNAs, regulate gene expression by post transcriptional binding and thereby suppressing protein translation. They are present in most eukaryotic cells and play an important role in nearly all physiological and regulative processes. Small RNAs were detected in various extracellular locations such as blood plasma and urine. However, very less information is available about the small RNA composition in biofluids such as milk and whether milk possesses its own defined small RNA profile differing from blood. To generate a holistic overview of all present small RNAs in bovine blood and milk and to identify shifts in their profiles, small RNA NGS was performed on whole blood and milk samples during the progressing lactation period. Small RNA-Sequencing was performed using an Illumina HiSeq and subsequent data analysis was done independently using either the GGS and GGA stations from Genomatix Software GmbH (Munich, Germany) or using freely available python scripts and R-packages (Bioconductor). First focus was on the dynamic regulation of microRNAs in milk in comparison to blood. Significantly regulation of miRNAs between different tissues and lactation stages was defined by a fold change of expression of at least 2-fold and a Benjamini-Hochberg adjusted p-value of less than 0.05. To validate these findings key microRNAs were quantified via RT-qPCR for fold change comparisons. Experimentally validated mRNA targets for regulated miRNAs were taken from the Tarbase 6.0 database from Diana Lab (Athens, Greece) and pathway analyses were generated using GePS (Genomatix Pathway System). RNA-sequencing clearly showed that milk possesses its own unique small RNA profile compared to blood and highlights its changes during the lactation period, with focus on the colostrum phase. Pathway analysis for affected targets revealed heavy influences on cell cycle progression, cell adhesion, DNA repair, apoptosis, and oncogenic defense. This underlines the potential role of microRNAs in mammary gland and milk not only for the mammary immune system, but also as an active modulator of gene expression in newborn calf.

Engineered Enzymes and Optimized Workflows for Next Generation Sequencing

Eric van der Walt, Maryke Appel, Gavin Rush, John Foskett, Paul McEwan

Kapa Biosystems, Woburn, MA, USA

Dramatic improvements in commercial Next Generation Sequencing (NGS) technologies have resulted in spectacular reductions in the cost-per-base of DNA sequencing. Until recently, the primary focus for innovation has been on improvements to the core sequencing technologies, with optimization of sample preparation playing a secondary role. The exponential gains in sequencing capacity have simultaneously led to higher sample throughput, placing increasing emphasis on the importance of improved library construction protocols for multiplexed sample sequencing and automation.

We have applied directed evolution and enzyme engineering to develop novel DNA polymerases that offer unique advantages for NGS library construction and quality control. We have employed these novel enzymes in optimized, automated, high throughput library construction and quality control protocols to produce high quality NGS libraries in a wide variety of applications and contexts.

We and others have previously reported on the advantages of an engineered, high-fidelity polymerase for library amplification, which enables improved library quality and sequence coverage. To extend the benefits of low bias amplification, particularly to low-input applications, we recently developed a library construction protocol that incorporates the "with-bead" strategy conceptualized by The Broad Institute of MIT and Harvard. Together with the use of ultra-pure, high quality reagents for library construction, the "with-bead" protocol results in significantly higher recoveries of adapter-ligated molecules from lower amounts of input DNA. These benefits allow for fewer cycles of amplification, thereby further reducing the risk of PCR-induced bias, error and other artifacts that can affect library quality, sequence coverage and reliable library quantification.

Comprehensive Biomarker Approach By Utilizing qPCR, NGS, in situ PCR: A Tool Box To Decipher Cancer

Robert P. Loewe

GeneWake GmbH, Germany

In many indications like oncology there is an increasing demand to stratify patient populations with reliable biomarkers to ensure effective therapies. Taking colorectal cancer as an example, multiple methods are demonstrated in a combinational approach to analyze biomarkers from different angles to provide a larger picture. After a short overview of routine methods like pathological examination, micro-dissection and purification of DNA/RNA, mutation analyses of known markers via next generation sequencing (NGS) and high resolution melting (HRM) are highlighted. Subsequently, gene expression analysis (mRNA and microRNA) and epigenetic analysis with its impact on cancer therapy are shown. Finally, the use of in situ PCR will be demonstrated, including the development of a highly innovative method to visualize mutations directly in formalin fixed tissue sections. Taken together, this combinational approach can help to understand complex patterns of biomarkers and the interaction of genes and also to evaluate and find those biomarkers that are useful as companion diagnostics or those that are therapeutically relevant for an improved and personalized treatment of cancer.

Less Than 80% Consensus On The Same Data - Comparative Variant Calling Of Pipelines And Replicates

Lu Zhang[1], Milena Kovacevic[2], Milos Popovic[2], <u>Sebastian Wernicke</u>[1]
[1]Seven Bridges Genomics, Cambridge, MA, USA; [2]Seven Bridges Genomics, Belgrade, Serbia

Background: Whole-exome sequencing has demonstrated remarkable success in identifying disease-causing genetic variation. In order to be applicable in clinical settings, however, consistency and accuracy of variant detection in sequencing data must be ensured. The same data set should yield the same variants.

Method: We compared the SNPs and indels (small insertions and deletions) detected by two variant calling pipelines and using two different sequencer runs on the same sample. Pipeline 1 was a "standard" BWA + GATK pipeline with all parameters, including subsequent filtering and region selection, conforming to the best practices described by The Broad Institute. Pipeline 2 employed the open source Mosaik aligner and the FreeBayes variant caller, both with best practice parameter settings recommended by the tool authors. Input data was the most sequenced human individual from the 1000 Genomes project, NA12878 (CEU), including two sets of exome pair-end sequencer runs, SRR292250 from an Illumina HiSeq 2000 and SRR088693 from an Illumina GA II. The identified exome variants were evaluated against a comprehensive set of QC metrics, including distribution of called variants relative to the dbSNP 135 database, SNP Transition to Transversion Ratio, as well as SNP and indel call set concordances between the replicates and pipelines.

Results: Both pipelines identified a high percentage of SNPs that were present in dbSNP 135 (99.73% and 97.49%, respectively); the consensus set had the highest concordance with known variants (99.80%). With BWA + GATK, the technical replicates resulted in a consensus of 21690 out of 22254 known SNPs, or 97.5%. The Mosaik + Freebayes pipeline identified 687 novel SNPs compared to 67 for the BWA+GATK pipeline; however, judging by Ti/Tv ratio, the former are more likely to be sequencing errors while the latter more likely to be "true" variants. The BWA + GATK pipeline identified 2112 indels with a high concordance to known indels of >91%, compared to 2457 indels with concordance >82% using Mosaik+Freebayes. As with the SNPs, the consensus set of Indels from both pipelines produced the highest concordance of over 95%. With BWA + GATK, the technical replicates resulted in a low consensus of only 889 out of 1147 indels, or 77.5% (BWA+GATK).

Conclusion: Although both variant calling pipelines achieved a relatively high accuracy on each dataset, each pipeline as well as each sequencer run produced a significant number of non-overlapping results. This demonstrates that the empirical choice of programs and parameters can miss important variants and contain false positives. While the accuracy of variant calls can be increased through a consensus of methods (as was done, e.g., in the 1000 Genomes Project), our analysis indicates that the current state of alignment and variant calling is not ready to standardize on a single tool. It will be imperative to ensure consensus results in order for NGS data to be clinically useful.

Lunch time seminar 1: Life Technologies - digital PCR

Time: **Tuesday, 19/Mar/2013: 1:00pm - 2:00pm**
Session Chair: **David N. Keys**, Life Technologies, United States of America

Location: **Lecture hall 14**
650 participants, TUM Weihenstephan

Presentation

Digital PCR Workshop with the QuantStudio™ 3D

<u>David N. Keys</u>

Life Technologies, United States of America

Digital PCR is an extremely promising method for increasing accuracy, precision and sensitivity of nucleic acid quantification beyond the capabilities of traditional real-time qPCR. This is done by combining PCR assays with single molecule sensitivity with a system to partition a sample into a set of reactions which number close to, or more than, the total number of target molecules in that sample. The combination of these functions makes it possible to calculate the number of target molecules present by counting the number of reactions with or without amplification. Many novel applications are enabled by this new approach, including reference free absolute quantification and high sensitivity rare-allele quantification. We will be presenting a live demonstration of the QuantStudio™ 3D Digital PCR System, a new digital PCR platform from Life Technologies™. This chip based system enables collection of up to 20,000 data points per sample run. The workflow has been optimized for simplicity, minimizing hands-on time, minimizing the risk of sample cross contamination, and minimizing sample loss. In this workshop, we will review the basics of digital PCR theory and practice, discuss the capabilities of the QuantStudio™ 3D Digital PCR System, and demonstrate the steps involved in a full digital PCR experiment, including chip loading, running, imaging and data analysis.

Lunch time seminar 2: Biogazelle - qbasePLUS data analysis

Time: **Tuesday, 19/Mar/2013: 1:00pm - 2:00pm**
Session Chair: **Barbara D'haene**, Biogazelle, Belgium

Location: **Lecture hall 15**
350 participants, TUM Weihenstephan

Presentation

qbase^PLUS to speed up the analysis of your qPCR data and to improve the accuracy of your experiments

<u>Barbara D'haene</u>

Biogazelle, Belgium

Are you struggling to get your qPCR data-analysis right? Do you want to speed up your analysis?
Join Barbara D'haene, PhD, for a lunch talk and get access to qbase^PLUS.
During this session Barbara will show how to analyse a qPCR experiment using qbase^PLUS. The key points demonstrated will be quality control, normalization and easy biostatistical analysis.
qbase^PLUS is based on the proven geNorm and qBase technology. The software is developed at Biogazelle by recognized qPCR experts Jo Vandesompele and Jan Hellemans. Biogazelle is a young and dynamic PCR company, eager to accelerate the discoveries in the PCR community.

Session Biomarkers: Transcriptional Biomarkers

Time: **Tuesday, 19/Mar/2013: 2:00pm - 6:30pm**
Session Chair: **Stephen Bustin**, Anglia Ruskin University, United Kingdom
Session Chair: **Massimo Bionaz**, Oregon State University, USA

Location: **Lecture hall 14**
650 participants, TUM Weihenstephan

Presentations

Systems Physiology in Cattle: Transcriptome Dynamics and Beyond

Massimo Bionaz[1], Juan J Loor[2]
[1]Oregon State University, United States of America; [2]University of Illinois at Urbana-Champaign

Genome sequencing efforts and the impressive development of technologies able to measure the cellular transcriptome with high accuracy have greatly enhanced the depth of research in livestock biosciences. The study of cattle physiology and nutrigenomics, in particular, has benefited from completion of the genome sequencing, ongoing annotation efforts, application of high-throughput technologies, and the development of more sophisticated bioinformatics approaches. Despite all those improvements, many challenges remain both ontological and methodological. Improper application of quantitative RT-PCR and inadequate use of statistics and bioinformatics for the analysis of large transcriptome datasets is still quite often observed in published research in cattle. The latter, in particular, limit the meaningful biological interpretation of omics data partly because of a persistent reductive approach to science. The reductionist approach has been challenged by the resurgence of the system biology approach. Such approach aims to study any system, from cells to entire organisms as they are: a complex system driven by dynamic interactive networks. Integrative systems biology is required to fully capture such dynamism. This approach is well-suited to study the complexity of cattle physiological adaptations to lactation, response to health challenges, and nutrition. The dynamic transcriptional adaptation across multiple tissue/organs, i.e., systems physiology, remains a major challenge. The Dynamic Impact Approach (DIA) appears suitable for the application of integrative system physiology. The DIA analysis of large transcriptome datasets allowed biological visualization of the dynamic adaptations of liver, mammary gland, and adipose tissue in dairy cows during the transition from pregnancy to lactation as well as the dynamic adaptation to nutrition. Interactive networks between gene products are fundamental for life; thus, their study is crucial to understand the biological adaptations of any biological system. Transcriptional network analysis has demonstrated great utility for understanding dynamic adaptations to physiological state or nutrition in dairy cows. Besides the transcriptome, including also non-coding short RNA, we are witnessing an ever-growing development of metabolomics and epigenomics technologies and, possibly, the development of bioinformatics tools able to integrate information from all those technologies. The future of integrative systems biology in dairy cattle biosciences holds great promises and appears very exciting!

An ORGANOTYPIC CULTURE MODEL for the STUDY of COLORECTAL CANCER.

Stephen Bustin

Postgraduate Medical Institute, Anglia Ruskin University, Chelmsford, UK

Studies aimed at elucidating the complex mechanisms driving colon cancer initiation, progression and therapeutics are hampered by the limitations of current models. Animal paradigms may not be wholly biologically relevant to human disease and cell cultures invariably have lost a number of specialised biochemical and behavioural properties of the parent tissues. We report the development of a long-term colorectal organotypic model based on multicellular cultures that mimic *in vivo* morphological features of neoplastic colonic mucosa. Colorectal cancer cell lines are cultured in a discrete environment consisting of a collagen matrix enclosing primary colonic fibroblasts, vascular smooth muscle and endothelial cells that is placed on a custom-designed support and suspended over growth medium. This approach approximates a three dimensional *in vitro* representation of the tumour microenvironment and results in the formation of large cellular aggregates that histologically resemble high-grade epithelial dysplasia or adenocarcinoma. Colon-specific differentiated structures develop in the constructs over a period of 21 days, with localised de-differentiation and evidence suggestive of epithelial-to-mesenchymal transition becoming apparent between days 28 and 56. Dissection of tissue sections allows the extraction and analysis of DNA, mRNA and miRNA from both the epithelial and stromal components of this model. When compared with two-dimensional adherent cell culture techniques, this protocol represents a more relevant system for the *in vitro*assessment of agents that regulate growth and progression in colorectal cancer.

Ion AmpliSeq[TM] Technology: A Tool For Biomarker Discovery And Beyond

David Ruff

Life Technologies, United States of America

Ion AmpliSeq[TM] technology is a simple, efficient and rapid process for enriching hundreds to thousands of targets for next generation sequencing. This technology is based on highly multiplexed single tube PCR amplification using either gDNA or cDNA templates. A great advantage for this approach is the small sample requirement for amplification reactions. As little as 1-10 ng of FFPE or whole genomic DNA can be readily amplified for Ion Personal Genome Machine® (PGM[TM]) sequencer runs. This chemistry is scaleable to very high levels of multiplex. The Ion AmpliSeq[TM] Comprehensive Cancer Gene Panel has been designed to include 4 pools of ~4,000 amplicons per tube. This panel covers the coding exon regions for 409 cancer genes. Another panel of ~10,000 amplicons distributed into 3 tubes covers 325 inherited disease gene exon coding regions. Moreover, the upper boundaries of plexity have yet to be reached – successful feasibility has been demonstrated at the 26,500-plex level for individual AmpliSeq reactions. Ion AmpliSeq[TM] technology is seamlessly applied to discover somatic biomarker mutations in cancer research samples. For example, we embarked on a collaborative effort to identify somatic biomarkers that may drive neoplastic evolution in esophageal cancer. The Ion AmpliSeq[TM] Custom Design pipeline was employed to layout 1395 primer sets for amplicons to cover the coding exons of 20 frequently mutated genes. Our study interrogated 40 tumor gDNA samples along with their matched normal counterparts and identified 21 novel somatic mutations. An emerging application for translational research is to access, detect and sequence circulating cell-free (ccf) nucleic acid biomarkers in human blood samples. We have a developed a workflow to purify and sequence ccf gDNA. Furthermore, we can also detect and quantify ccf mRNA using a multiplex preamplification scheme. And finally, employing a novel mRNA-expression capture method, we report a breakthrough approach that enables detection and analysis of newly synthesized mRNA species. This tool provides an avenue to identify and quantify real-time transcriptional biomarker activity in cellular responses. In summary, highly multiplexed PCR technology powers the Ion AmpliSeq[TM] platform and this combination promises to bring forth a next generation landscape that will redefine the boundaries of biomarker discovery.

For Research Use Only. Not for Use in Diagnostic Procedures.

A Kinetic PCR Model Function and its Application on the LightCycler® 96

Rolf Knobel

Roche Diagnostics International, Switzerland

For the automatic analysis of kinetic PCR data the regression of a mathematical curve function to the measurement signals appears attractive. The derived curve function is robust against signal imprecision and curve characteristics like a Cq value can be calculated easily. However, simple mathematical models like the logistic or Richard function fail to globally fit real kinetic PCR data well in many cases. Here, a novel mathematical function for kinetic PCR curve regression is introduced. It shows appropriate asymptotic behavior and can deal with different curvatures in the increase and the saturation phase of the reaction. Additionally, the application of this function for automatic PCR result calculation is shown: The calculation of the asymptotic signal growth size is useful for the correction of the spectral crosstalk of cleaved TaqMan products. A drifting baseline can be derived from the mathematical model curve as well in order to standardize the parallel curve display of many curves measured in different wells. For Cq calculation the intersection of a threshold line with the model function is insensitive to signal imprecision. Additionally, an automated qualitative judgment based on several curve characteristics like growth and maximal slope is possible. With the help of pre-set algorithm cutoffs the automatic calculation of the results can be adjusted for different applications like intercalation or multicolor TaqMan on each detection channel of an instrument. For the LightCycler® 96 thousands of real data sets have been used for training the algorithm parameters accordingly. So, the result calculation of kinetic PCR reactions can be performed fully automatic in a reliable way. LightCycler® is a trademark of Roche.

Transcriptional Profiling To Address Molecular Determinants Of Endometrial Receptivity – Lessons From Studies In Livestock Species

Susanne E. Ulbrich[1], Anna E. Groebner[1], Stefan Bauersachs[2]

[1]Physiology Weihenstephan, Technische Universität München, Freising, Germany; [2]Laboratory for Functional Genome Analysis (LAFUGA), Gene Center, LMU Munich, Munich, Germany

The development of a fertilized oocyte into a differentiated multi-cellular organism is a major challenge with regard to the orchestration of the expression of the mammalian genome. Highly complex networks of genes are temporally and spatially regulated during cellular differentiation to generate specific cell types. Embryonic development is critically influenced by external impacts in the female reproductive tract. A most critical phase of pregnancy in mammals is the pre- and peri-implantation period, during which the uterine environment plays a crucial role in supporting the development of the conceptus. The analytical description of the transcriptome, proteome and metabolome of the embryo-maternal interface is a prerequisite for the understanding of the complex regulatory processes taking place during this time. This review lines out potentials and limitations of different approaches to unravel the determinants of endometrial receptivity in cattle, the pig and the horse. Suitable in vivo and in vitro models, which have been used to elucidate factors participating in the embryo-maternal dialog are discussed. Taken together, transcriptome analyses and specified selective candidate gene driven approaches contribute to the understanding of endometrial function. The endometrium as sensor and driver of fertility may indicate the qualitative and quantitative nature of signaling molecules sent by the early embryo and in turn, accordingly impact on embryonic development.

Candidate Blood Transcriptomic Markers of Early Onset Major Depression Derived from Etiological Animal Models of Depression

Eva E. Redei[1], William Gardner[2,3], Andrea Luis[2], Brandon Strange[3], Kathleen Pajer[2]

[1]Northwestern University Feinberg School of Medicine, United States of America; [2]Department of Psychiatry, Dalhousie University Faculty of Medicine, Canada; [3]Department of Pediatrics, Ohio State University College of Medicine, Columbus, OH, USA

Early onset Major Depressive Disorder (MDD) is a serious and prevalent psychiatric illness in adolescents and young adults. Current treatments are not universally effective. Biological markers of early onset MDD could increase diagnostic specificity, but no such biomarker exists. Our innovative approach to biomarker discovery for early onset MDD combined results from genome-wide transcriptomic profiles in the blood of two animal models of depression, representing the genetic and the environmental, stress-related, etiology of MDD. The genetic animal model is bred by bidirectional selection in a test for depressive behavior from the accepted animal model of depression, the Wistar Kyoto rat. The "depressed" WMI (Wistar Kyoto More Immobile) strain responds to tricyclic antidepressants and MAOIs by normalized behavior, and has dysfunctions in connectivity in the brain similar to human depressed patients. The genetically close Wistar Kyoto Less Immobile (WLI) strain serves as the non-depressed control. Transcriptomic differences between these strains, that showed the same directional expression differences in the blood as in the hippocampus or amygdala, were selected as potential blood markers for MDD. The chronic stress model employed four genetically distinct strains of rats and established the transcriptional consequences of chronic stress in the blood regardless of genotype or vulnerability to stress. Some of these chronic stress-related transcripts were also selected as candidate blood markers for MDD.

We carried out unbiased analyses of the 26 candidate blood transcriptomic markers, selected from the two models, in a sample of 15-19-year-old subjects with MDD and subjects with no disorder (ND). A panel of 11 blood markers differentiated participants with early onset MDD from the ND group. Additionally, a separate, but partially overlapping panel of 18 transcripts distinguished subjects with MDD with or without comorbid anxiety. Based on an interacting protein network analysis, transcripts were differentiated as markers of MDD alone, MDD and anxiety and anxiety alone, suggesting overlapping and separate molecular mechanisms for MDD with and without comorbid anxiety. Four transcripts, based on the chronic stress animal model, correlated with maltreatment scores in youths. Thus, our approach can lead to clinically valid diagnostic panels of blood transcripts for early onset MDD, which could reduce diagnostic heterogeneity in this population and has the potential to advance individualized treatment strategies.

Novel Solution Enables Automation of Data Analysis and QC of Real-Time PCR Diagnostics

Aron Cohen, Ze'ev Russak, Martine Bernstein

Azure PCR, United Kingdom

Infectious diseases provide enormous challenges to all healthcare systems and highlight the need for medical devices that improve diagnosis as well as tracking disease in real-time; two fundamental tools which the Azure PCR strategy aims to integrate.

qPCR is used in the detection of infection because of its ability to identify disease and quantify pathogens and with $2.8bn of sales, qPCR remains the most commonly used platform in molecular diagnostics. However there are problems with the conventional methods adopted in the analysis of qPCR data. Current automated methods can yield inaccurate results and thresholds need to be set manually even when using specialist software. Utilisation of such software requires local expertise, introduces inaccuracies and as such data analysis is difficult to standardise and increases data interpretation costs. Additionally, threshold-based methods can incorrectly classify sample curves by calling false positives/negatives.

The Azure PCR solution overcomes the current limitations of conventional qPCR data analysis methods by offering a high-throughput,

standardised system that uses machine learning techniques to automate analysis of qPCR clinical data for molecular diagnostics. The technology can also process complex multiplex melt-curve data for multiple mutation and infection testing. Characteristics of the qPCR curves such as the transition points are automatically determined to create a unique 'fingerprint,' without any manipulation of raw data. The technology has been proven to work in close to 100,000 analyses, with at least 99% accuracy achieved in comparison to manual analysis. Data is archived and results are instantly accessible for QA and tailored user reports are created. The solution can interface with both internal and external systems and is available in both online and offline versions

In addition, Azure PCR are currently optimising collation of data from collaborators with the intention of developing a cloud-based approach to disease surveillance that automatically aggregates the data. Azure PCR aim to produce high quality health statistics in real-time that are distributed globally to aid the planning and implementation of health policies in all countries.

Azure PCR performs qPCR data analysis without requiring any threshold settings or training for the operator, thus our technology allows access to qPCR in new, previously unrealisable settings and development of tests (e.g. personalised medicine) which previously would have been too complex to analyse. Automation will also allow development of diagnostic tools that can be operated in a point of care environment providing swift diagnosis to patients.

Thus we offer an innovative system for molecular diagnostic automation and standardisation including methods for analytical assay validation and global tracking and surveillance of infectious disease.

A workflow for the isolation and molecular characterization of Individual Circulating Tumor Cells (CTCs) to enable cell heterogeneity analysis and personalized therapy

Gianni Medoro, <u>Francesca Fontana</u>, Alex Calanca, Chiara Bolognesi, Stefano Gianni, Maximilian Sergio, Giulia Bregola, Anna Doffini, Giulio Signorini, Antonino Catania, Manuela Banzi, Elena Peruzzi, Giuseppe Giorgini, Nicolò Manaresi

Silicon Biosystems, Italy

Study of rare cells has become increasingly attractive in various areas of basic and translational bio-medical research, drug discovery and advanced diagnostics. In oncology, molecular characterization of Circulating Tumor Cells (CTCs) is one of the main application areas so far. CTCs hold the promise to deliver an up-to date picture of the patient mutational profile, which is key for therapy selection in the framework of personalized therapy.

The purification of cells which, like CTCs, have a frequency as low as 1 in a billion (10^{-9}) within heterogeneous suspension of other cells, is the critical issue. Due to the low numbers and the possible heterogeneity of the target cells themselves, a highly automated method to reliably isolate 100% pure cells with single cell resolution would be ideal. While significant progresses have been achieved in the enrichment methods, no platform so far had been able to fill the gap from enriched populations to single pure cells. Another key issue comes downstream of cell isolation, as the low amount of DNA or RNA included in a single cell needs to be amplified in a highly balanced and accurate way to reflect the original content of a single cell in order to allow meaningful molecular profiling.

We show that DEPArray™, an automated, image-based sorting platform, achieves the goal of isolating 100% pure cells with single cell resolution from enriched suspensions. It employs the principle of dielectrophoresis to individually trap and move fluorescently labeled cells on a microelectronic chip with over 300,000 programmable electrodes in a digital, deterministic and highly controlled way.

Furthermore, using Ampli1™ WGA, a method of whole genome amplification from single cells based on ligation mediated PCR, we demonstrate molecular characterization of single CTCs from cancer patients, including mutation detection, genotyping and next generation sequencing analysis. We show that digitizing the sample (i.e. analyzing pure single CTCs collected by DEPArray™) and amplifying the DNA with Ampli1™ WGA it is possible to tap into the power of Next Generation Sequencing (using targeted semiconductor based sequencing with IonTorrent® PGM), overcoming the sensitivity – specificity trade-off which otherwise prevent their use due to the sequencing noise.

Experiments assessing cell viability, when working with live cells will also be presented. The potential impact on translational oncology research will be discussed, as the opportunity to move from simple counting of CTC further deep into CTC or Cancer Stem Cells (CSC) characterization, offers an unprecedented insight into a tumor development and a unique opportunity to develop accurate companion diagnostics and personalized therapies.

Session HT & OPT: High Throughput Analysis & qPCR Optimization

Time: **Tuesday, 19/Mar/2013: 2:00pm - 6:30pm**
Session Chair: **Jan Hellemans**, Biogazelle, Belgium
Session Chair: **Patricia de Winter**, UCL, United Kingdom

Location: **Lecture hall 15**
350 participants, TUM Weihenstephan

Presentations

Insights from the first RT-qPCR based human transcriptome profiling based on wet lab validated assays.

<u>Jan Hellemans</u>, Pieter Mestdagh, Barbara D'haene, Ariane Deganck, Jo Vandesompele

Biogazelle, Belgium

The MIQE guidelines describe the assay parameters to be evaluated in the lab before giving green light on a qPCR assay for use in quantification studies. Wet lab validation of a qPCR assay appears to be a challenge for some users, or an unwanted extra burden for many others. We will describe the procedures by which we have successfully completed the full wet-lab validation of RT-qPCR assays for all protein coding genes of the human and mouse transcriptome. One of the key improvements in our validation approach is the use of massively parallel amplicon sequencing for an unprecedented stringency on the assay specificity.

After extensive validation, we have used all human assays (n=18,841) on the four MicroArray Quality Control (MAQC) RNA samples to obtain the first RT-qPCR based human transcriptome expression profile. The data reveal an impressive dynamic range with 90% of protein coding genes detected over a 20 million fold range. In addition, this study enables a thorough comparison of various quantification performance aspects of qPCR with those of microarrays and RNA sequencing obtained in the MAQC and SEQC studies, respectively. Finally, this study yields insights in the usefulness of the qPCR Reference RNA sample as positive control and another view on the publicly available data of the genes expressed in brain.

Overcoming PCR inhibition: Next Generation qPCR and RT-qPCR ToughMix® Reagents for High Sensitivity Quantification and Accurate Genotyping from Crude Samples.

David Mark Schuster

Quanta BioSciences, United States of America

Naturally occurring PCR-inhibitory components can reduce the sensitivity, efficiency or specificity of PCR assays and gives rise to false negatives and allele dropouts. Reported PCR inhibitors include compounds such as hemin (or its derivatives), bile salts, vitreous fluid, aromatic halides, melanin, complex carbohydrates, polyphenolic compounds from plant extracts, and humic substances produced during the decomposition of organic matter. While the most effective approach to prevent PCR inhibition is to remove the inhibitor from the sample, this is not always certain. Often inhibitors may co-purify with the DNA sample. Effective nucleic acid extraction/purification methods are expensive, time consuming, and often result in a loss of material. Implementation of high throughput, cost effective DNA analysis by PCR therefore demands robust amplification reagents that can relieve PCR inhibition. This talk will present the development of new qPCR reagents that overcome many reported PCR inhibitors: PerfeCta qPCR ToughMix, AccuStart Genotyping ToughMix, and qScript XLT One-Step RT-qPCR ToughMix.

Rapid qPCR using a novel Taq mutant

Patricia de Winter[1], David Sugden[2]

[1]UCL, United Kingdom; [2]KCL, United Kingdom

High throughput qPCR is achievable by automation of sample processing and preparation of reactions, but the rate-limiting factor is the long cycling time required by most chemistries. Although hardware capable of very fast cycling has been available for a number of years, the DNA polymerase in the master mix, typically requires more than 10 seconds to anneal to the target template and extend the product (amplicon). Previously we routinely used a 3-step qPCR protocol in a Rotor-Gene 6000, an air-driven instrument generally capable of shorter cycling times compared with block-based machines. We recently tested a new mutant Taq (formulated as Brilliant III Ultra-Fast SYBR green qPCR mix, Agilent) reported to allow an extremely short combined annealing and extension step. Available data performed on plate-based real-time cyclers examined only two target genes. In this presentation we report the use of Brilliant III to amplify over 150 targets with a wide range of amplicon sizes from various species, and its resistance to PCR inhibitors. Using 5 sec denaturation (95°C) and 1 sec combined annealing/extension (57°C) for 40 cycles, standards of known copy number prepared from purified PCR products (qStandard) exhibited high efficiency ($\geq$98%), and excellent linearity between 10^7 to 10^1 copies (r^2 >0.999). Serially diluted cDNA and gDNA amplified with similarly good efficiency and linearity. Even challenging templates such as NGS libraries, which contain multiple amplicons were readily amplified. The effect of amplicon length on amplification efficiency and linearity was determined. Four common PCR inhibitors did not diminish Brilliant III performance even at high concentrations. The very rapid amplification enabled by the remarkably short annealing/extension step reduced total cycling time to ~39 min without compromising assay efficiency and linearity, indicating that excellent performance can be achieved routinely with Brilliant III.

A Nū Solution for Real-Time Quantitative PCR

Gothami Padmabandu

Illumina Inc, United States of America

Rreal-time quantitative PCR (RT-qPCR) has been widely used in the analysis of gene expression for decades. Currently many probe based chemistries have been utilized for RT-qPCR analysis. NūPCR reagents constitute a novel probe-based technology that utilizes a unique NūZyme chemistry. NūZymes are multipart catalytic enzymes that recognize and assemble on target nucleic acid sequences forming a catalytic complex. This three part probe consists of two PartZyme oligos and a universal substrate oligo containing a 5' flourophore and a 3' quencher that binds to the partzyme oligo. Once the target is amplified via PCR, the two partzymes bind to the specific target sequence forming the NūZyme complex. The enzymatic activity of the NūZyme cleaves the fluorescently labeled universal substrate producing a signal that can be detected by a real-time PCR instrument.

The high sensitivity, specificity and ease of designing duplexed assays will be presented. Different applications of NūPCR such as a highly multiplexed DNA quantitation assay for forensic samples will be discussed.

Twisted Intercalating Nucleic Acid (TINA) – a novel group of molecules with improved performance in PCR and qPCR applications

Rainer Schubbert

Eurofins Medigenomix, Germany

The recently developed TINA molecule (Twisted Intercalating Nucleic Acid) from QuantiBact A/S enhances the thermal stability of a binding oligonucleotide duplex while leaving the ability to discriminate matching and mismatching oligonucleotides intact. TINA labeling increases the primer annealing and melting temperature and therefore reduces the general probability of PCR primers to anneal unspecific.

This increase in specific primer annealing can be utilized to increase the overall specificity of a given assay. Alternatively, by "relaxing" the stringency of the primer annealing, an increased sensitivity can be achieved without compromising specificity compared to an identical assay without TINA-modifications.

We present examples from routine analyses (food authenticity, residual DNA, pathogen detection), where we have validated whether TINA modified oligos allows better discrimination in allele specific PCR and RealTime PCR assays or higher sensitivity in RealTime PCR assays compared to analyses with unmodified oligos.

Automated PCR Setup for High Throughput Analysis Enabled by the Labcyte Echo® 525 Liquid Handler and Access™ Workstation

Celeste Glazer, Carl Jarman

Labcyte, United States of America

The Echo 525 liquid handler enables PCR assay setup for real-time quantitative PCR (qPCR), SNP analysis and DNA sequencing setup in reaction volumes from 10 µL to as little as 250 nL. The Echo liquid handler eliminates the risk of cross contamination and the requirement to pre-dilute samples or PCR reagents, enabling PCR setup and optimization in 384- and 1536-well microplates with greater efficiency. The Access workstation combines the Echo liquid handler with a range of devices (including systems for qPCR analysis) into flexible and modular solutions targeted for walk away, cost-effective high throughput PCR setup. This tutorial discusses validation of the Echo Liquid Handler for PCR setup and the implementation of an Access workstation for fully automated processing.

Factors influencing the transfer of multiplex assays between qPCR instruments

Ossian Saris

Thermo Fisher Scientific, Vantaa, Finland

Multiplex reactions offer several advantages over singleplex reactions. Combining several targets into the same reaction brings substantial time and reagent savings. Furthermore the detection of more than one sequence within the same reaction vessel allows control reactions to be included. Internal controls can be used to detect reaction inhibition, which may cause false negative results.

Optimizing multiplex reactions brings several challenges due to the complex interaction of several primers and probes. Amplicons compete for the available reagents, such as primers, polymerase and nucleotides. Other issues include designing of primers in a way to avoid primer dimer formation and the optimization of primer concentrations to enable several targets to be amplified in the same reaction.

Multiplexing qPCR reaction also requires careful choice of fluorescent dyes and matching quenchers. Choosing the correct dyes to fit qPCR instruments may be difficult, because all instruments contain unique excitation wavelengths and filters for both excitation and emission. However, most instruments come with a pre-calibrated dye set and usually with the possibility to calibrate additional dyes.

In publications, laborious process of optimizing multiplex reactions is often performed with a single instrument and reagent. Replicating the results with a different instrumentation and reagents often results in varying degree of success. Aside from the differences between instrument detection channel wavelengths, not much is known about the transfer of multiplex assays from one instrument to another, representing an interesting topic to study.

ValidPrime questions the need for DNase treatment in RT-qPCR experiments

Henrik Laurell[1], Jason Iacovoni[1], Jean-José Maoret[1], Jean-François Arnal[1], Mikael Kubista[2]

[1]Inserm / Université Paul Sabatier UMR1048, Institut des Maladies Métaboliques et Cardiovasculaires (I2MC), BP84225, 31432 Toulouse cedex 4, France; [2]TATAA Biocenter AB, Göteborg, Sweden

Genomic DNA (gDNA) contamination is an inherent problem during RNA purification, which can lead to non-specific amplification and aberrant results in reverse transcription (RT)-qPCR. gDNA sensitivity for most qPCR assays can be greatly diminished by appropriate precautions during assay design. However, regardless of the primer design strategy, the inability of a Gene-Of-Interest (GOI) assay to amplify gDNA needs to be validated experimentally. ValidPrime offers this possibility. Prior to ValidPrime, RT-minus controls were the only option available to evaluate the impact of the gDNA background on RT-PCR data. ValidPrime measures the gDNA contribution using an optimized gDNA-specific ValidPrime assay (VPA), targeting a non-transcribed locus, and gDNA reference sample(s) that permit normalization for GOI-specific differences in gDNA sensitivity. The RNA-derived component of the signal can be accurately estimated and deduced from the total signal. ValidPrime corrects with high precision for gDNA, up to 60% of the total signal, while substantially reducing the number of required qPCR control reactions.

In conclusion, ValidPrime 1) offers a cost-efficient alternative to RT(-) controls; 2) accurately validates qPCR assays in terms of their sensitivity to gDNA 3) allows correction for gDNA-derived signals; 4) reduces the need for DNase treatment 5) The dedicated ValidPrime software greatly simplifies the analysis.

Correction of RT-qPCR data for genomic DNA-derived signals with ValidPrime. Laurell H, Iacovoni JS, Abot A, Svec D, Maoret JJ, Arnal JF, Kubista M. Nucleic Acids Res. 2012 April 1;40(7):e51.

Session MIQE: MIQE & QC strategies in qPCR

Time: **Wednesday, 20/Mar/2013: 8:30am - 12:30pm**
Session Chair: **Gregory L Shipley**, Shipley Consulting, LLC, USA
Session Chair: **Afif Michel Abdel Nour**, KAU/KFRMC, Saudi Arabia

Location: **Lecture hall 14**
650 participants, TUM Weihenstephan

Presentations

MIQE 2009-2013 - its impact four years after publication

Stephen Bustin

Postgraduate Medical Institute, Anglia Ruskin University, Chelmsford, UK

Approximately four years that have passed since the publication of the MIQE guidelines, allowing an objective assessment of their impact on qPCR-based research. The picture is rather mixed: on the plus-side, the MIQE paper itself is seeing significantly increased month-on-month citation rates and there has been and continues to be widespread publicity around MIQE, with numerous web seminars, workshops and information leaflets spreading the message of transparency and standardisation. In addition, the keen engagement by many instrument and reagent manufacturers, led by BioRad and Agilent, has been a very positive development, resulting in a very high level of expertise amongst their field application specialists. How well researchers implement the MIQE guidelines is another issue, and here the picture is far less positive. Not all manufacturers take the guidelines seriously and many researchers, especially at the principal investigation level, are perfectly content to continue publishing data of questionable biological relevance. Most frustratingly, the editors of most high impact factor journals have not seen the need to encourage the use of the guidelines by their contributors, the BMC group of journals being the honourable exception. Ultimately, the technical standard of scientific publications will not increase until there is some incentive to follow guidelines and, although areas of qPCR-based research continue to spread, improved reagents are launched and analysis methods are becoming increasingly sophisticated, the quality of the research output remains suspect.

Quality control in Quantitative PCR

Kristina Lind, Jennifer Pettersson, Robert Sjöback, Mikael Kubista

TATAA Biocenter, Sweden

When working with qPCR it is very important to have control over all the different steps that are included in the process. It reaches from the experimental design all the way to the data analysis. Leaving out a part of the process from your quality control may result in erronous conclusions and and decisions. To help researchers and reviewers to have a better overview of the different parts that needs to be taken into consideration, the MIQE guidelines have been published. One important part is the qPCR assay itself. In this talk I will describe the process of validating new qPCR assays, how to determine efficiency, linear dynamic range, limit of detecion (LOD), limit of quantification (LOQ) and precision.

Applying The MIQE guidelines to clinical and pre-clinical trials

Maxime Dooms[2], Abalo Chango[2], Essam Azhar[1], Steve Harakeh[1], Elie Barbour[3], Flore Depeint[2], Afif Michel Abdel Nour[1]

[1]KAU/KFRMC/ Special Infectious Agent unit Biosafety Level 3, Saudi Arabia; [2]Institut Polytechnique LaSalle Beauvais, Beauvais, France;[3]American University of Beirut, Beirut, Lebanon

The ''Minimum Information for the Publication of qPCR Experiments'' guidelines are targeted at gene expression experiments and have to our knowledge not been applied to qPCR assays carried out in the context of clinical trials. This report details the use of the MIQE qPCR app for iPhone (App Store, Apple) to assess the MIQE compliance of one clinical and five pre-clinical trials. This resulted in the need to include 14 modifications that make the guidelines more relevant for the assessment of this special type of application. We also discuss the need for flexibility, since while some parameters increase experimental quality, they also require more reagents and more time, which is not always feasible in a clinical setting. The second part of my talk will be an update on the MIQE-qPCR app through numbers since 2011. I will finish by a quick review on the implementation of the MIQE guideline in the Middle East challenges and opportunities.

Applying the MIQE Guidelines to Screens Utilizing qPCR Focused Arrays

Gregory L Shipley

Shipley Consulting, LLC, United States of America

Performing a screening experiment utilizing qPCR and focused miRNA or mRNA plate arrays is not like running a standard qPCR experiment. Experimental design and execution require some compromises that immediately precludes the implementation of the full set of recommendations as outlined in the MIQE Guidelines. In this talk I will show the differences between the two experimental procedures and how best to setup qPCR focused array screening experiments utilizing automation. I will then discuss post-screen validation of screening results, present some tips on running these experiments and show it's importance in completing a MIQE compliant screening experiment.

Management and Automation of qPCR Diagnostic Workflows

Matjaz Hren

BioSistemika, Slovenia

In the molecular diagnostics field, qPCR (Real-Time Polymerase Chain Reaction) is one of the leading methods because it allows detection and precise quantification of specific DNA / RNA sequences. The core advantages that ensure qPCR's broad applicability are high sensitivity and specificity and a broad dynamic range. Sample preparation and data analysis are complex qPCR steps, mostly performed by high-level experts. There are several solutions available for automation of the so called "wet-lab" part of the workflow (which includes sample preparation, reagent & sample loading onto qPCR plates). However when it comes to the so called "dry-lab", which covers experiment design, data analysis and interpretation, quality control and reporting, there are no simple solutions available. In addition to that there are no internationally accepted guidelines or standards for diagnostic use of qPCR such as MIQE for research qPCR community.

This problem can be solved by different approaches. One of the most common is in-house development of solutions by preparing a series of macro-based spreadsheet documents in combination with modifications of Laboratory Information Management Systems (LIMS) to automate as much of the qPCR workflow as possible. However users still have to use different program environments to design analyses, create templates for lab work (e.g. mastermix calculation) and finally to analyse and interpret results and to prepare reports. This approach is still quite tedious and includes some repetitive work, which is somewhat contradictory to the demand for obtaining fast results without compromising quality in qPCR diagnostics. So we see that these solutions only partially address the demand for automation, unification and simplification of the qPCR workflow in diagnostic environments. Therefore we designed easy to use software that manages complete diagnostic qPCR workflow. The software guides users from the initial experiment design, to the final reports utilizing powerful expert knowledge. This easy to use software unifies the entire qPCR workflow. It calculates reagent concentrations for selected analyses, prepares wet lab outputs, analyses raw data and interprets the results while taking into account the hierarchical positions among lab employees. It communicates with LIMS and qPCR thermal cyclers and is quality assurance compliant. It has been designed to turn complex and unconsolidated approach into one simple, controlled environment. Its basic purpose is easing the pressure on high-level scientists and to make qPCR diagnostics and efficient process.

The use and usefulness of amplification curve analysis in quantitative PCR.

Jan M Ruijter[1], Michael W Pfaffl[2], Sheng Zhao[3], Andrej N Spiess[4], Gregory Boggy[5], Jochen Blom[6], Robert G Rutledge[7], Davide Sisti[8], Antoon Lievens[9], Katleen De Preter[10], Stefaan Derveaux[11], Jan Hellemans[12], Jo Vandesompele[10]

[1]Academic Medical Centre, Amsterdam, the Netherlands; [2]Technical University of Munich, Weihenstephan, Germany; [3]University of California, Berkeley, USA; [4]University Hospital Hamburg-Eppendorf, Germany; [5]eDNA Software Inc., Ann Arbor, USA; [6]Center for Biotechnology, Bielefeld University, Germany; [7]Laurentian Forestry Centre, Quebec, Canada; [8]University of Urbino, Urbino, Italy; [9]Department of Applied Mathematics and Computer Science, Ghent, Belgium; [10]Center for Medical Genetics, Ghent, Belgium; [11]Wafergen, Fremont, CA, USA; [12]Biogazelle, Zwijnaarde, Belgium

RNA transcripts such as mRNA or microRNA are frequently used as biomarkers to determine disease state or response to therapy. Reverse transcription (RT) in combination with quantitative PCR (qPCR) has become the method of choice to quantify small amounts of such RNA molecules. In parallel with the democratization of RT-qPCR and its increasing use in biomedical research or biomarker discovery, we witnessed a growth in the number of gene expression data analysis methods. Most of these methods are based on the principle that the position of the amplification curve with respect to the cycle-axis is a measure for the initial target quantity: the later the curve, the lower the target quantity. However, most methods differ in the mathematical algorithms used to determine this position, as well as in the way the efficiency of the PCR reaction (the fold increase of product per cycle) is determined and applied in the calculations. Moreover, there is dispute about whether the PCR efficiency is constant or continuously decreasing. Together this has lead to the development of different methods to analyze amplification curves. In published comparisons of these methods, available algorithms were typically applied in a restricted or outdated way, which does not do them justice. Therefore, we aimed at development of a framework for robust and unbiased assessment of curve analysis performance whereby various publicly available curve analysis methods were thoroughly compared using a previously published large clinical data set. The original developers of these methods applied their algorithms and are co-author on this study. We assessed the curve analysis methods' impact on transcriptional biomarker identification in terms of expression level, statistical significance, and patient-classification accuracy. The concentration series per gene, together with data sets from unpublished technical performance experiments, were analyzed in order to assess the algorithms' precision, bias, and resolution. While large differences exist between methods when considering the technical performance experiments, most methods perform relatively well on the biomarker data. The data and the analysis results per method are made available to serve as benchmark for further development and evaluation of qPCR curve analysis methods (http://qPCRDataMethods.hfrc.nl).

Four Years of RDML qPCR Data Format – Achievements and Opportunities

Andreas Untergasser[1], Steve Lefever[2], Jan M Ruijter[3], Jan Hellemans[4], Jo Vandesompele[2,4]

[1]University Heidelberg, Heidelberg, Germany; [2]Ghent University, Ghent, Belgium; [3]Academic Medical Center, Amsterdam, The Netherlands;[4]Biogazelle, Zwijnaarde, Belgium

Quantitative PCR (qPCR) is the gold standard method for accurate and sensitive nucleic acid quantification. To improve the quality and transparency of experiment design, data-analysis and reporting of results, the MIQE guidelines were established in 2009 (Bustin et al., Clinical Chemistry). One of the recommended items was making the raw qPCR data available under the form of a universal data format.

The Real-time PCR Data Markup Language (RDML) was designed to establish a vendor independent, freely available XML based file format to store and exchange qPCR data. RDML stores the raw data acquired by the machine as well as the information required for its interpretation, such as sample annotation, primer and probe sequences and cycling protocol. When provided with publications, RDML-files should enable readers to re-evaluate the data and confirm the conclusions. The first version of RDML was published in 2009 and has already been cited 50 times. Furthermore, the RDML file format was supported by instrument manufacturers realizing its potential and today Bio-Rad (CFX96 and CFX384), Life Technologies (StepOne, ViiA7 and QuantStudio) and Roche (LC96) have enabled their instrument software to export data in the RDML-format. Additionally, third party software supporting RDML has started to emerge. The software solutions include primer design tools (primer3plus), assay databases (RTPrimerDB) and data analysis software (LinRegPCR and qbasePLUS). Even though more and more qPCR instruments are able to store data in the RDML format and RDML is being increasingly used in instrument independent data analysis, still too few publications make raw data available in the RDML format.

As qPCR continues to develop, so does RDML. The latter development is coordinated by the RDML consortium, a group of scientists, software developers and instrument manufacturers (http://www.rdml.org). The joined efforts resulted in an improved 1.1 version, and version 1.2 is currently being drafted. This consortium is not limited to its current members; it invites all interested parties to join the effort. [On behalf of the RDML consortium]

The Impact of MIQE Guidelines in the Plant Science Community.

Ellen De Keyser, Laurence Desmet, Jan De Riek

ILVO, Belgium

Currently in plant research, validated reliable RT-qPCR protocols are still rare. The last decade gene expression studies have been implemented widely in plant science. However, the methods used are often only semi-quantitative or quantification was not at all performed according to the MIQE-guidelines. The necessity of using multiple reference genes has become obvious in quite some cases, but assay-specific validation of these genes is often lacking. Still too often reference genes are selected for a species-wide application, no matter what treatment is given. Also RNA quality control is a crucial bottleneck. Machines for capillary electrophoresis allow to determine RNA quality quite easily, but how do you deal with this information? RIN or RQI values do not apply on plant material, since the training software was only developed using human/animal material. Plant material does not contain a 28SrRNA band but a 25S band. In addition, total RNA in chloroplast-containing plant tissues also consists of 16S and 23S rRNA adding 2 extra peaks. An alternative approach (visual evaluation) needs to be taken to decide on the quality of plant RNA samples. The use of noRT samples is another delicate point. Hardly any paper reports on the use of noRTs and in those cases noRTs were used after all, no information is available on the outcome. In our experience, the appearance of samples of which the Cq-value of the noRT is within 5 units of the actual sample are common in most experiments. Ignoring this information can lead to a severe overestimation of the gene expression in a specific sample. These three problems will be discussed more profoundly in view of the necessary application of the MIQE-guidelines in plant research.

Session non-coding RNAs: microRNA, siRNA and long non-coding RNAs

Time: **Wednesday, 20/Mar/2013: 8:30am - 1:00pm**
Session Chair: **Pieter Mestdagh**, Ghent University, Belgium
Session Chair: **Mirco Castoldi**, Universitätsklinikum Düsseldorf, Germany

Location: **Lecture hall 15**
350 participants, TUM Weihenstephan

Presentations

Evaluation of quantitative microRNA gene expression platforms in the microRNA Quality Control (miRQC) study

Pieter Mestdagh[1], Toumy Gettouche[2], Thomas Peters[3], Nicole Hartmann[3], Jo Vandesompele[1]

[1]Ghent University / Biogazelle, Belgium; [2]University of Miami, Florida, USA; [3]Novartis Institutes for BioMedical Research, Novartis, Basel, Switzerland

MicroRNAs are important negative regulators of protein coding gene expression, and have been studied intensively over the last few years. To this purpose, different measurement platforms to determine their RNA abundance levels in biological samples have been developed. In this study, we have systematically compared 12 commercially available microRNA expression platforms by measuring an identical set of 23 standardized positive and negative control samples, including human universal reference RNA, human brain RNA and titrations thereof, human serum samples, and synthetic spikes from homologous microRNA family members. We developed novel quality metrics in order to objectively assess platform performance of very different technologies such as small RNA sequencing, RT-qPCR and (microarray) hybridization. We assessed reproducibility, sensitivity, quantitative performance, and specificity. The results indicate that each method has its strengths and weaknesses, which helps guiding informed selection of a quantitative microRNA gene expression platform in function of particular study goals.

[abstract presented on behalf of the miRQC consortium]

Non-Coding RNAs in Tumor and Inflammatory Diseases.

Jörg Hackermüller[1,2,3], The Ribolution Project Consortium[4]

[1]Young Investigators Group Bioinformatics and Transcriptomics, Helmholtz Centre for Environmental Research - UFZ, Leipzig, Germany;[2]RNomics group, Fraunhofer IZI, Leipzig, Germany; [3]Department of Computer Science, University of Leipzig, Germany; [4]www.ribolution.org

Large-scale transcriptome projects such as ENCODE and FANTOM have dramatically changed our view of mammalian genome organization: The bulk of the genome is transcribed – but most of these transcripts do not code for proteins. Many of these non-protein coding RNAs (ncRNAs), in particular long ncRNAs, display specific expression patterns that depended strongly on cell type, tissue, and developmental timing, hinting at a functional role of these transcripts and suggesting their use as biomarkers.

The R & D consortium "Ribolution - Innovative Ribonucleic acid-based Diagnostic Solutions for Personalized Medicine" aims at identifying and validating short and long non-coding and coding RNA biomarkers for various diseases. Ribolution strongly relies on deep strand specific transcriptome sequencing, followed by validation in larger cohorts using custom arrays and qPCR. For chronic obstructive pulmonary disease (COPD) Ribolution aims at whole blood biomarkers that predict disease progression, characterized by decline of lung function. In prostate carcinoma the consortium develops biomarkers for the discrimination of aggressive versus indolent forms of the disease. In summary, our results support the concept that long non-coding RNA expression in blood and tissue samples is a promising source of diagnostic and prognostic biomarkers.

An Optimized miRNA Profiling System for Limiting Samples

__Jonathan Michael Shaffer__

QIAGEN, United States of America

microRNAs (miRNAs) are naturally occurring, small noncoding RNAs that mediate post-transcriptional gene regulation in many biological processes including differentiation and development, cell signaling, and response to infection. Overwhelming evidence also indicates that dysregulation of miRNA expression is a cause or indicator of several disease processes, including many cancers, and that their expression varies as a result of disease. As a result, miRNA expression signatures are being pursued as biomarkers in disease diagnosis and prevention. Biomarker development, however, relies heavily on samples that may contain limited amounts of RNA including FFPE, serum, plasma, and other body fluid samples. In addition, straightforward workflows or tailored solutions (based on tissue or sample type) aren't readily available to aid in streamlined miRNA biomarker development. Success of miRNA biomarker development relies on overcoming these hurdles, and this is accomplished using QIAGEN's complete wet-bench solutions and recommended workflows. Specifically, the miRNeasy kits enable isolation of high-quality total RNA from virtually all samples types. Following isolation, the miScript PCR System, consisting of the miScript II RT Kit, miScript PreAMP PCR Kit, miScript miRNA PCR Arrays, and data analysis software, enables accurate, comprehensive miRNome expression profiling by removing the limiting sample barrier. Regardless of sample type or RNA amount, miRNA biomarker development is within reach as a result of the high performance cataloged and customizable research tools and solutions available from QIAGEN.

Emerging role of blood circulating microRNA as non-invasive biomarker

__Mirco Castoldi__

Universitätsklinikum Düsseldorf, Germany

One of the major challenges towards an improved treatment of human diseases is the identification of appropriate markers for an early detection of diseases as well as for monitoring disease progression and patients' response to therapy. Cell-free nucleic acids circulating in human blood were first described in 1948. However, it was not until 1994 that the importance of circulating nucleic acid (CNA) was recognized. Today, the detection of diverse type of CNA in blood and other body fluids is a valuable resource in the search for novel biomarker. Recently, cell-free miRNA have been isolated and measured in the blood. The existence of a population of circulating miRNAs (CiMs) in the blood of healthy as well as diseased individuals have raised the possibility that disease-associated CiM signatures may serve as biomarker in the diagnosis, prognosis, and prediction of therapeutics response of patients. Importantly, CiM can be detected by using different experimental approaches including quantitative real-time PCR, microarray and Next Generation Sequencing (NGS). With this premise, circulating miRNAs have been 'tagged' by the biomedical community as easily accessible biomarkers for the screening of patients. To ensure a reliable and reproducible measurement of CiM in human blood, we have established optimized isolation protocols, which can be directly implemented in clinical settings. Our current research work in this field focuses on the identification of CiM specifically associated to viral hepatitis, leukemia and asthma, and on the correlation of CiM-signatures with the severity of the disease.

Generating Robust Results From qPCR Analysis Of MicroRNAs In Biofluids

__Ditte Andreasen__, Thorarinn Blondal, Maria Wrang Theilum, Niels Tolstrup, Jörg Krummheur, Nana Jacobsen, Peter Mouritzen

Exiqon A/S, Denmark

microRNAs (miRNAs) constitute a class of small cellular RNAs (typically 19-23 nt) that function as post-transcriptional regulators of gene expression. Current estimates indicate that more than one third of the cellular transcriptome is regulated by miRNAs, although they are relatively few in number (less than 2000 human miRNAs).

The high relative stability of miRNAs in common clinical source materials (FFPE blocks, plasma, serum, urine, saliva, etc.) and the ability of microRNA expression profiles to accurately classify discrete tissue types and specific disease states have positioned microRNAs as promising new biomarkers for diagnostic application. Furthermore miRNAs have been shown to be actively exported from tissues into the circulation with the development of pathology, through a variety of mechanisms including exosome and microvesicle transport, and complexing with RNA binding proteins or HDL.

The main challenge in detecting miRNA is their small size, no longer than one standard PCR primer. Furthermore, many miRNAs belong to families with only a few mismatches between members, and the G/C content varies greatly. To overcome these obstacles and facilitate discovery and clinical development of miRNA-based biomarkers in biofluids, we developed an LNA™-based miRNA PCR platform with unparalleled sensitivity and robustness. The platform uses a single universal RT reaction per sample combined with two target specific, LNA™ enhanced PCR primers per miRNA assay to profile human miRNAs. This facilitates high-throughput profiling of miRNAs from important clinical sources, with high specificity and sensitivity, without the need for pre-amplification. We have recently further developed the technology with a new RNA extraction kit and improved qPCR reagents, further increasing sensitivity and specificity while allowing high-throughput processing with the use of ambient temperature stacking systems.

Using the LNA™ PCR system, we have profiled thousands of biofluid samples. An extensive QC system has been implemented in order to secure technical excellence and reveal any unwanted bias in the dataset. We will present our approaches to sample handling, qPCR technology, data normalization and studies of pre-analytical variables such as hemolysis.

Integration of disparate sources of information to predict miRNA-mRNA interactions

Ander Muniategui[^1], Ignacio Sanchez-Caballero[^2], Rubén Nogales-Cadenas[2], Carlos O. Sánchez-Sorzano[2], Alberto Pascual-Montano[*2],__Angel Rubio[*1]__

[1]CEIT & TECNUN, University of Navarra, Spain; [2]Funciotnal Bioinformatics group, CNB-CSIC, Madrid, Spain

miRNAs are small RNA molecules (9 22nt) that interact with their corresponding target mRNAs inhibiting the translation of the mRNA into proteins and cleaving the target mRNA. This second effect diminishes the overall expression of the target mRNA. Several miRNA-mRNA relationship databases have been deployed, most of them based on sequence complementarities. However, the number of false positives in these databases is large and they do not overlap completely. In many cases the researcher has not clue on which database is the most suitable for his/her needs. Usually, the union of the databases is used in order to avoid missing possible interactions. This approach has an

important drawback: all the databases are considered to be equally reliable (and is not the case). In some cases, it is also interesting to combine miRNA and mRNA expression with the sequence based-predictions to achieve more accurate relationships.

In this work, we present a method that estimates the reliability of each interaction that appear in the databases. This method aggregates the information of the different databases using a Bayes approach. Using this information, we have created a metadatabase that ouperforms published databases in both extension and quality. This database can be combined with mRNA and miRNA expression to find specific interactions that appear in a experiment. To this end, we used weighted LASSO regression with non-positive constraints (Talasso).

We show that the suggested metadatabase provides a meaningful score and that the top-ranked interactions are more enriched in experimentally validated interactions than any other database. In addition, this score can be combined with Talasso to get interactions that actually appear in a particular experiment.

TaLasso is available as Matlab or R code. There is also a web-based tool for human miRNAs at http://talasso.cnb.csic.es/.

How to Narrow Down the Complexity of Possible miRNA and mRNA Interactions in Cellular Differentiation?

Swanhild U Meyer[1], Steffen Sass[2], Fabian J Theis[2], Michael W Pfaffl[1]

[1]Physiology Weihenstephan, ZIEL Research Center for Nutrition and Food Sciences; [2]MIPS, Institute for Bioinformatics and System Biology, Helmholtz Center Munich, German Research Center for Environmental Health, Neuherberg, Germany

Background - *In silico* target prediction of miRNAs often reveals several thousand possible mRNA interactions for an individual miRNA. Elucidating the function of miRNAs by target prediction algorithms is biased by false positives and negatives as well as miRNA targets which might not be relevant in a specific cellular context. However, holistic miRNA-target interaction analysis by cross-linking and pull-down experiments is time consuming and expensive. Using miRNA and mRNA expression data in combination with *in silico* predictions allows improving the chance of predicting functional relevant miRNA-mRNA interactions.

Methodology / Principal Findings - We used samples of *in vitro* myoblast differentiation and differentiation with TNFalpha or IGF1 treatment for profiling analysis of miRNA (Agilent microarray, Life Technologies qPCR card) and mRNA (Affymetrix array) expression. For integrated miRNA-mRNA analysis 21 miRNAs were selected. We used the data of each miRNA profiling platform as well as a joint dataset of microarrays and qPCR cards representing the intersection of both platforms. Target prediction was based on TargetScan (www.targetscan.org) and miRanda (www.microrna.org) data. Integrated miRNA-mRNA was performed by miRLastic, a negative multiple linear regression analysis. miRLastic analysis including the intersection miRNA dataset (individual miRNA datasets) revealed around 3,800 (6,700) putative miRNA target interactions in total and about 180 (320) targets per miRNA on average. Target prediction solely based on *in silico* data by e.g. miRanda would have resulted in more than 127,000 total target predictions and about 6,000 targets per miRNA. For focusing on predicted miRNA-mRNA interactions with a high likelihood of being functional in myogenic differentiation we applied filter criteria such as the number of inverse miRNA-target correlations of a given miRNA, number of transcription factors targeted per miRNA, number of different miRNAs targeting a gene transcript, as well as enrichment of targets by cocitation or in GO terms and signalling pathways.

Conclusions - To narrow down the complexity of possible miRNA and mRNA interactions in myogenic differentiation we integrated the analysis of miRNA and mRNA profiling data and target prediction algorithms. The resulting number of possible miRNA target interactions was further reduced by the sequential application of specific selection criteria to generate a dataset of high putative functional significance.

High-throughput lncRNA expression profiling identifies candidate cancer lncRNAs

Pieter Mestdagh[1], Steve Lefever[1], Kristina Althoff[2], Carina Leonelli[1], Jan Hellemans[3], Marine Jean-Christophe[4], Johannes Schulte[2], Jo Vandesompele[1]

[1]Center for Medical Genetics, Ghent University, Belgium; [2]Department of Pediatric-Oncology, University Hospital Essen, Germany; [3]Biogazelle, Ghent, Belgium; [4]VIB Laboratory for Molecular Cancer Biology, Leuven, Belgium

Recent studies suggest that our genome is pervasively transcribed and produces many tens of thousands of long non-coding RNAs (lncRNAs). These lncRNAs have been implicated in gene expression regulation through direct interaction with chromatin modifying complexes and their subsequent recruitment to target loci in the genome. To date, only a handful of lncRNAs have been described with dcoumented functions in cancer biology. However, their critical role as regulators of gene expression suggests that lncRNAs, much like microRNAs, might be key components of different cancer pathways.

In order to study lncRNAs in cancer, we designed and extensively validated a high-throughput RT-qPCR lncRNA expression profiling platform capable of quantifying over 1700 lncRNAs in a single run. This platform has been applied to identify lncRNAs downstream of two major cancer genes, MYC and TP53, by means of inducible model systems. Furthermore, we measured lncRNA expression of the entire NCI60 cancer cell line panel.

Both MYC and TP53 were found to directly induce lncRNA expression through direct promoter binding. The findings were validated in primary samples and model systems of different tumour entities. From the NCI60 panel, we identified cancer specific lncRNA signatures, reminiscent of lineage survival oncogene expression patterns. Together, our results suggest that RT-qPCR is a valid screening approach for high-throughput lncRNA quantification revealing multiple candidate cancer lncRNAs.

Lunch time seminar 3:   Genomatix – Next Generation Sequencing data analysis

Time: **Wednesday, 20/Mar/2013: 1:00pm - 2:00pm**
Session Chair: **Christian Zinser**, Genomatix Software GmbH, Germany

Location: **Lecture hall 14**
650 participants, TUM Weihenstephan

Presentation

Start Making Sense – NGS Data Analysis with Genomatix

Christian Zinser

Genomatix Software GmbH, Germany

The ongoing evolution of Next Generation Sequencing is revealing ever more of the complexity of genomes, gene expression, and gene regulation. To harness the considerable potential of this technology for creating biologically relevant information, data generation must be matched by analysis tools and strategies which integrate and consolidate the available lines of evidence into scientifically interpretable results.

This talk provides an overview of the solutions offered by Genomatix for the analysis of Next Generation Sequencing data.

The presentation will focus on the following analysis methods:

Assessment of genomic variants; Expression and transcript fusion analysis; Examination of regulatory features: protein-DNA binding and DNA methylation; Data integration employing positional correlation, genome annotation, biological classification, regulatory pathways, and gene networks

Examples will include the application of the above to the study of cancer, the identification of the basis of hereditary diseases, and the elucidation of disease-relevant regulatory networks.

Lunch time seminar 4:   MultiD - GenEx data analysis seminar

Time: **Wednesday, 20/Mar/2013: 1:00pm - 2:00pm**
Session Chair: **Mikael Kubista**, TATAA Biocenter, Sweden
Session Chair: **Amin Forootan**, Multid Analyses AB, Sweden

Location: **Lecture hall 15**
350 participants, TUM Weihenstephan

Presentation

GenEx – the ultimate tool for qPCR data analysis

Mikael Kubista[1,2]**, Amin Forootan**[2,3]**, Björn Sjögren**[3,4]
[1]TATAA Biocenter, Sweden; [2]MultiD Analyses AB; [3]Goteborg University; [4]Lawrence Livermore National Laboratory

As qPCR is becoming mature technology with widespread use requirements on validation, quality assessment and reporting increases. This is particularly important when data are submitted for approval by regulatory bodies, reported to clients, or published in quality journals. For molecular diagnostic applications this includes determining PCR efficiency with confidence intervals, establishing the linear range of the assay, its limit of quantification, limit of detection and random error. For routine applications also estimates of repeatability and reproducibility may be relevant. For the samples analyzed estimated target concentrations shall be indicated with confidence intervals. These analyses are recommended by the Clinical and Laboratory Standards Institute. In my presentation I will show how these analyses are performed on qPCR using GenEx.

Goal of expression profiling is to explain biological phenomena. Workflow starts by planning, designing and optimizing an experiment, collecting the data, analyzing the data, and extracting biological information. Typically large amounts of data are collected that are batch imported and pre-processed to remove variation between runs, reduce intersubject variation, and minimize technical noise. Missing data are also handled. The data are then analyzed using powerful multivariate statistical methods including hierarchical clustering, principal component analysis (PCA), and self-organized maps. Dynamic PCA is used for variable selection to identify the most relevant genes explaining the observations. Finally, the data are passed from GenEx for cloud based pathway analysis with the Ingenuity iReport. In will present seamless workflow from the collection of data to the extraction of biological information using GenEx

www.multid.se   www.tataa.com   www.clsi.org   www.ingenuity.com

Session SC: Single-Cell Diagnostics

Time: **Wednesday, 20/Mar/2013: 2:00pm - 4:30pm**
Session Chair: **Kenneth James Livak**, Fluidigm Corporation, USA
Session Chair: **Anders Ståhlberg**, University of Gothenburg, Sweden

Location: **Lecture hall 14**
650 participants, TUM Weihenstephan

Presentations

A Microfluidic Device that Isolates Single Cells then Processes RNA for qPCR or Sequencing

Kenneth James Livak
Fluidigm Corporation, United States of America

The stochastic nature of generating eukaryotic transcripts challenges conventional methods for obtaining and analyzing single-cell gene expression data. As part of a study exploring the effect of genotype on Wnt pathway activation, data were collected for 96 qPCR assays on individual cells from 15 genotyped lymphoblastoid cell lines (HapMap lines). For each cell line, qPCR was used to obtain single-cell gene expression profiles for 48 baseline cells and 48 stimulated cells. Thus, data were collected from a total of 1440 single cells. The methods used in the collection and analysis of single-cell qPCR data will be contrasted with those used in conventional qPCR. The generation of RNA libraries from hundreds to thousands of single cells has been simplified by introduction of the C_1™ Single-Cell Autoprep System (Fluidigm). This system captures up to 96 single cells and performs the processing steps of cell lysis, cDNA synthesis by reverse transcriptase, and initial amplification to generate libraries for qPCR or RNA-Seq analysis.

Genome Analysis Of Individual Cells

Christian Korfhage
QIAGEN GmbH, Germany

DNA sequence analysis and genotyping of biological samples using next-generation sequencing (NGS), microarrays, or real-time PCR is often limited by the small amount of sample available. A single cell comprises only one to four copies of the genomic DNA, depending on the organism (haploid or diploid organism) and the cell cycle phase. The DNA amount of a single cell ranges from a few femtograms in bacteria to picograms in mammalia. In contrast, a deep analysis of the genome requires a few hundred nanograms up to micrograms of genomic DNA. Consequently, accurate whole genome amplification (WGA) of single cell DNA is required for reliable genetic analysis (e.g., NGS) and is particularly important when genomic DNA is limited, as in single cell WGA. The use of single-cell WGA has enabled the analysis of genomic heterogeneity of individual cells (e.g., somatic genomic variation in tumor cells).

To perform single cell WGA, we used the QIAGEN® REPLI-g® Single Cell Kit, which uses a method based on isothermal multiple displacement amplification (MDA). This technique is capable of accurate in vitro DNA replication of a single whole genome directly from single cells due to innovative lysis and the use of an optimized form of the Phi 29 Polymerase with:

(1) Proofreading activity (up to 1000-fold lower error rates compared to *Taq* polymerase), (2) Strong processivity (resulting in minimal enzyme dissociation at difficult structures such as GC rich regions), (3) Strong DNA displacement activity (resulting in solving hairpin structures).

Here, we describe the reliability of this single cell WGA method and its application to next-generation sequencing (NGS) and real-time PCR.

Quantitative PCR Analysis of DNA, RNAs, and Proteins in the Same Single Cell

Anders Ståhlberg

University of Gothenburg, Sweden

The single cell represents the basic unit of all organisms. Most investigations have been performed on large cell populations, but understanding cell dynamics and heterogeneity requires single-cell analysis. Current methods for single-cell analysis generally can detect only one class of analytes. Reverse transcription and the proximity ligation assay were coupled with quantitative PCR and used to quantify any combination of DNA, mRNAs, microRNAs (miRNAs), noncoding RNAs (ncRNAs), and proteins from the same single cell. The method has been demonstrated on transiently transfected human cells to determine the intracellular concentrations of plasmids, their transcribed mRNAs, translated proteins, and downstream RNA targets. We developed a whole-cell lysis buffer to release unfractionated DNA, RNA, and proteins that would not degrade any detectable analyte or inhibit the assay. The dynamic range, analytical sensitivity, and specificity for quantifying DNA, mRNAs, miRNAs, ncRNAs, and proteins were shown to be accurate down to the single-cell level. Correlation studies revealed that the intracellular concentrations of plasmids and their transcribed mRNAs were correlated only moderately with translated protein concentrations (Spearman correlation coefficient, 0.37 and 0.31, respectively; $P < 0.01$). In addition, an ectopically expressed gene affected the correlations between analytes and this gene, which is related to gene regulation. This method is compatible with most cell-sampling approaches, and generates output for the same parameter for all measured analytes, a feature facilitating comparative data analysis. This approach should open up new avenues in molecular diagnostics for detailed correlation studies of multiple and different classes of analytes at the single-cell level.We will also discuss how single-cell data can be used to gain detailed information about cell types and cell states.

Visualizing gene expression at the single cell, single chromosome, single RNA, and single base level

Marshall Levesque, Arjun Raj

Biosearch Technologies Inc., United States of America

We use fluorescence in situ hybridization to provide highly specific and direct detection of individual RNA molecules via fluorescence microscopy in their natural context in cells and tissues. This method enables absolute quantification of gene expression in single cells, and we discuss how to interpret these measurements. We highlight the detection and localization of long non-coding RNAs. We also have extended our assay to detect chromosome structure and gene expression at the same time, enabling per-chromosome transcriptional profiling. We also present a method that allows us to measure single-base differences on individual RNA molecules.

Single-Cell Digital Gene Expression On Up To 800 Unique Transcripts Using Optically-Barcoded Single-Nucleic Acid Counting: Comparison With Microfluidic qPCR And RNA-seq (Whole Transcriptome)

Michael Rhodes

Nanostring Technologies, United Kingdom

True digital (i.e., counting based) multiplexed gene expression can currently only be performed using RNA-seq or optically-barcoded single-nucleic-acid counting (nCounter technology). We demonstrate that a simple modification to the nCounter protocol enables digital quantification of 800 unique transcripts in a single cell, offers several potential advantages relative to single-cell microfluidic PCR, and much better counting statistics than single-cell (or normal input) RNA-seq (when compared to whole transcriptome). The nCounter Single Cell protocol incorporates reverse transcription and linear pre-amplification (10 to 18 cycles) with a highly multiplexed pool of up to 800 gene-specific primer pairs in a single tube, followed by hybridization with optically-barcoded nucleic-acid-labels. Microfluidic qPCR methods require the same pre-amplification step, but must be followed by splitting the amplified sample into 96 separate wells and performing an additional series of up to 40 PCR amplification cycles. nCounter technology requires no sample-splitting (a true multiplex) or additional amplification cycles and (compared to RNA-seq) doesn't require library generation because single-molecules are counted directly. Gene-expression measurements of flow-sorted single-cells using nCounter, revealed the stochastic "on-off" behavior. The "summed" (aggregate) gene expression profile from multiple individual flow-sorted cells was (essentially) identical to pools of multiple flow-sorted cells (10 per tube and 100 per tube), proving digital linearity of 800-targets at the single-cell level for the first time. When comparing to RNA-seq (whole transcriptome), nCounter (on panels of 100's-of-genes) resolved a constant ~ 2 million on-target reads per sample (~10,000X coverage), compared with < 100,000 on-target reads for RNA-seq (as expected for a non-targeted approach). Hundreds-to-thousands of single-cells, 800 targets-each, can be examined per-week on an nCounter system: enabling single-cell digital biology.

Session MD 2:   Molecular Diagnostics 2

Time: **Wednesday, 20/Mar/2013: 2:00pm - 4:30pm**
Session Chair: **Steve Lefever**, Ghent University, Belgium
Session Chair: **Irmgard Riedmaier**, TUM Physiology, Germany

Location: **Lecture hall 15**
350 participants, TUM Weihenstephan

Presentations

Cost-effective and robust genotyping using double-mismatch allele-specific quantitative PCR

Steve Lefever[1], Ali Rihani[1], Filip Pattyn[1], Tom Van Maerken[1], Jan Hellemans[2], Jo Vandesompele[1,2]
[1]Center for Medical Genetics Ghent, Ghent University, Ghent, Belgium; [2]Biogazelle, Zwijnaarde, Belgium

Although allele-specific PCR has been around for many years, its adoption for SNP genotyping has been hampered by its low discriminating power and the need for post-PCR gel electrophoretic analysis. In this study, we combine the basic principles of allele-specific PCR with the straightforward readout of SYBR Green I based qPCR technology, thereby eliminating the need for probes (either labeled or not) and reducing post-analysis time to a minimum. To further enhance the robustness and discriminating power of the method, an artificial mismatch in the allele-specific primer was introduced, resulting in a new type of assays coined double-mismatch allele-specific qPCR (DMAS-qPCR) assays. Our assays outperform hydrolysis probe based assays when looking at calling success rate (100 % vs. 96 % call rate, for 12 SNP tested on 48 samples) and can be used with sample input as low as 250 pg. The ease of use, the availability of online software for assay design, the absence of labeled probes and the high sensitivity of DMAS-qPCR are characteristic of a cost-efficient and powerful new way of SNP genotyping.

Assessment of Transcriptional Activity of *Borrelia burgdorferi* and Host Cytokine Genes During Early and Late Infection in a Mouse Model

Emir Hodzic

University of California at Davis, United States of America

Differential gene expressions by *Borrelia burgdorferi* spirochetes during mammalian infection and host cytokines genes were assessed. Despite the recent advances in gene transcription detection technology, there has been limited transcriptional analysis of large clusters of *B. burgdorferi* genes. In an initial survey to understand the global effect of host immune response on gene transcription of *B. burgdorferi* during infection in the mouse model, expression profiles of 43 different genes were selected, including those presumed to be involved in attachment, cell envelope, metabolism, complement regulation, cellular processes, and replication. We used a RT-qPCR based method on the low-density array (LDA) format to compare transcriptional levels of selected *B. burgdorferi* genes and 19 mouse chemokines and cytokines in mice during early (3 weeks) and late (2 months) infection. The amounts of *B. burgdorferi* RNA and cDNA available from collected mouse tissues are limited, which restricts the number of analyzable genes. To overcome this problem, a preamplification technique was used to enhance sensitivity and fidelity of the RT-qPCR, especially for low-abundance target genes that increases fidelity of target genes that can be analyzed. LDA represents a valuable approach for sensitive and quantitative gene transcription profiling for understanding this important vector-borne disease.

Is lung cancer genetic heterogeneity responsible for resistance to EGFR tyrosine kinase inhibitors? Contribution of digital PCR

Pascale Tomasini, Veronique Secq, Isabelle Nanni, Antoine Carlioz, Fabrice Barlesi, L'Houcine Ouafik, Frederic Fina

Assistance Publique Hôpitaux de Marseille, France

Introduction: Activating EGFR mutations are predictive biomarkers for a good response and survival to EGFR tyrosine kinase inhibitors (EGFR-TKI). However, ultimately, every patient progress. Several resistance mechanisms have been described, among which the concept of tumor heterogeneity is one of the most important. We studied tumor heterogeneity of the most common gene alterations described for lung cancer, and their role in resistance to EGFR-TKI. Patients and methods: On the one hand, 20 patients with concomitant KRAS and EGFR mutations routinely diagnosed in our laboratory were selected. These mutations were confirmed with qPCR-HRM and Sanger sequencing for each available section and block. EGFR mutations heterogeneity on blocks surfaces were studied using IHC. Deep biopsy cores were performed for EGFR and KRAS mutations heterogeneity sequencing analysis. The percentage of each EGFR and KRAS mutation was assessed using nano-droplets digital PCR (ddPCR ™). On the other hand, 7 EGFR-TKI-resistant patients with activating EGFR mutation were selected for qPCR- HRM and sequencing study of all molecular resistance factors known to date. Results: EGFR and KRAS mutations were different for each patient's block or section studied. EGFR mutations IHC labelling is heterogeneous and well correlated with molecular biology results. Deep biopsy cores analysis shows a greater heterogeneity for KRAS mutations versus EGFR mutations. For each sample, mutation rates are different for KRAS and/or EGFR as shown using ddPCR. Discussion: This study demonstrates the concept of tumor heterogeneity in a clinical context using highly sensitive techniques. These results also support the hypothesis for the existence of several tumor clones within with a selection and adaptation mechanism under treatments pressure.

Cost-effective real-time analysis by mediator probe (RT-)PCR

Simon Wadle[1], Stefanie Rubenwolf[1], Michael Lehnert[1], Bernd Faltin[2], Roland Zengerle[1,3,4], Felix von Stetten[1,3,4]

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Probe-based real-time PCR is used in molecular diagnostics due to its superior specificity and clinical sensitivity. High synthesis costs for sequence-specific dual-labelled detection probes are still one reason why researchers are reluctant when larger numbers of probes need to be ordered. In order to reduce costs we suggested a novel real time PCR method, the mediator probe PCR [1, 2]. It replaces fluorescently labeled hydrolysis probes by sequence-specific label free mediator probes (MP). Cleavage of the MP during amplification results in release of a mediator which is detected by a universal fluorogenic reporter (UR) oligonucleotide. The key to cost savings is that the same UR can be used for all assays and therefore can be ordered in large scale. This way oligonucleotide synthesis costs can be reduced to less than 40 % compared to the synthesis costs in hydrolysis probe based assays. In this work, performance characteristics of mediator probe PCR (MP PCR) were compared to hydrolysis probe PCR (HP PCR). Method: Nucleic acids were extracted from two viral DNA targets (human papilloma virus-18, human adenovirus B7) and one viral RNA target (influenza virus B). MPs and the UR were designed according to design rules provided in [1]. Probe sequences of the MPs were based on the corresponding HP sequences and comprise the following differences: (1) HP: fluorescence quencher at the 3'-terminus; MP: phosphate group at the 3' terminus to avoid extension by polymerase activity; (2) HP: fluorophore at the 5'-terminus; MP: mediator sequence at the 5' terminus. As the mediator and the corresponding mediator hybridization sites at the UR can be standardized the same fluorogenic UR could be used for all assays. Results: Analysis of serial dilutions of the DNA- and RNA targets revealed good agreement between MP and HP PCR: HPV18 (MP PCR r^2 = 0.999/HP PCR r^2 = 0.975), HAdV B7 (0.992/0.983), FluB (0.992/0.991). Amplification of 10^2 to 10^6 copies HPV 18 DNA per reaction gave CVs of 55.1% to 9.9% / 38.3% to 10.7% and accuracies in the range of +21.6% to -8.1% / +19.4% to -9.8%. Amplification of 10^2 to 10^5 copies HAdV B7 DNA per reaction gave CVs of 29.4% to 3.4% / 51.3% to 5.4% and accuracies ranging from +30.6% to -18.3% / +49.3% to -18.1%. Amplification of 10^4 to 10^7 copies FluB RNA per reaction gave CVs of 46.9% to 4.0% / 42.4% (4 out of 5 detectable) to 4.2% and accuracies ranging from +5% to -5% / +11% to -5%. Using the novel MP (RT-)PCR detection of 3 different targets with one universal detection oligonucleotide, the UR, showed same performance characteristics as HP (RT-)PCRs. MP PCR can reduce oligonucleotide synthesis costs and is recommended where a multitude of probes with low batch size is required. This can be in research laboratories, during assay development or in low resource settings. Our current research aims at (1) extending the design rules and (2) increasing the degree of multiplexing.

Somatic mutations – detecting less than 0.05% KRAS and BRAF mutation in a background of wildtype DNA

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By using modified oligonucleotides, it is possible to obtain unprecedented sensitivity when detecting somatic mutations. We will show how the use and optimization of SuPrimers™ (DNA primers with increased specificity), BaseBlockers™ (oligos suppressing amplification of wildtype genes) and HydrolEasy probes (hydrolysis probes with increased signal-to-noise ratio and sensitivity) in combination can yield assays with a sensitivity and specificity great enough to routinely detect less than 0.05% of mutated KRAS and BRAF in a DNA samples.

Abstracts – Poster Presentations

All posters will be displayed at all three poster sessions, starting on Monday evening 6 p.m. until Wednesday early afternoon 2 p.m.

Poser Session 1:   Monday Evening
Time: Monday, 18/Mar/2013:   6:00pm - 10:00pm
Location: Foyer - lower level

Poster Session 2:   Tuesday Lunch Time
Time: Tuesday, 19/Mar/2013:   12:30pm - 2:00pm
Location: Foyer - lower level

Poster Session 3:   Wednesday Lunch Time
Time: Wednesday, 20/Mar/2013:   12:30pm - 2:00pm
Location: Foyer - lower level

Next Generation Sequencing
P001 – P016

P001

Exome Sequencing To Detect Novel Mutations Causing Malignant Hyperthermia

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Malignant Hyperthermia (MH) is an autosomal dominant pharmacogenetic disorder which is potentially lethal. MH is triggered in susceptible individuals through exposure to volatile anesthetics during general anesthesia. During an MH episode, the myoplasmic calcium level increases rapidly due to increased flux of calcium from the sarcoplasmic reticulum (SR) to the cytosol. This results in a hypermetabolic state characterized by hyperthermia, muscle rigidity, tachycardia, hypoxaemia, and metabolic acidosis, which if untreated, can lead to death.

The primary locus for MH is the ryanodine receptor (*RYR1*, skeletal muscle calcium-release channel). In approximately 70% of MH-susceptible families mutations are found in *RYR1*, in 1% of cases mutations are found in *CACNA1S* (alpha 1S subunit of the voltage-dependent L type calcium channel). This indicates that MH-susceptibility in around 30% of cases is not linked to *RYR1* or *CACNA1S*.

We have sequenced the Exomes of three patients of an extended New Zealand family. We achieved 70x coverage and identified on average 12,000 non-synonymous changes in each patient. Non-parametric Linkage analysis suggests that MH in this family is linked to chromosomes 6 and/or 12. We excluded common polymorphisms and are now using High Resolution Melting (HRM) analysis to examine segregation of new or rare variants with the disease in the family.

P002

Exploring the Complex Transcriptome of Pigmented and Non-pigmented Bovine Skin Using RNAseq

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By generating a comprehensive transcriptomic landscape of cells and tissues, deep RNA sequencing (RNAseq) has opened a new horizon for understanding global gene expression. Using this approach, we performed a whole transcriptome analysis on bovine skin to describe the complex transcript catalogue of this tissue. Therefore, total RNA isolated from pigmented and non-pigmented skin areas from the same animals was subjected to deep mRNAseq analysis. The libraries generated by TruSeq mRNA library preparation were sequenced by a paired-end multiplex 2x 61 cycle run on an Illumina GAIIx sequencer. A total of 51,646 unique transcripts were mapped to the bovine reference genome assembly (UMD3.1, Ensembl release 66) using Bowtie/Tophat mapping tools. The majority of them could be mapped to known transcripts (68%) including a high proportion of transcripts displaying novel splice junctions (36%). In addition, a substantial number of novel transcripts (10,884; 30%) were discovered, which had not been annotated before. RNAseq data analysis using a pipeline for classification of unknown transcripts based on coding potential prediction as well as homology search and comparative sequence analysisin diverse RNA databases identified those transcripts, which had a high coding potential and orthologs in other species as novel bovine genes/transcripts. The classification analysis of unknown transcripts showed that RNAseq can also contribute to improve the annotation of known genes by providing evidence for additional exons, untranslated regions and splice variants and by confirming structural models of genes that have been in silico predicted. RNAseq data analysis revealed that the majority of unknown transcripts discovered in bovine skin could be assigned to non-coding RNA (ncRNA) types. Predominantly, they were mapped in intergenic chromosome regions and therefore, they could be classified as potential intergenic ncRNA. A fraction of the unknown transcripts could be classified as potential long ncRNA, based on interspecies conservation. Expression profiling analysis of all mapped transcripts showed that 677 loci were significantly (q<0.05) differentially expressed between pigmented and non-pigmented skin. Whereas many of those transcripts belong to the known melanin synthesis pathway, also genes relevant for immune response and intra- and intercellular trafficking were affected. However, there are also several examples for unknown transcripts with no homology to known genes from other species but different expression in pigmented vs. non-pigmented skin, which indicates that annotating and studying the function of ncRNA remain a major future challenge. The results of our study demonstrate a complex transcript pattern for bovine skin, suggest a functional relevance of novel transcripts in the modulation of pigmentation processes and highlight novel functional candidate genes that may affect the modulation of mammalian pigmentation.

P003

A Broad Re-sequencing Study Of 409 Genes In NCI-60 Cell Lines Using The Ion AmpliSeq™ Comprehensive Cancer Panel And Ion PGM™ Semiconductor Sequencing Reveals Previously Unreported Cell Line-specific Mutations

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Highly multiplexed PCR is quickly becoming the new template preparation paradigm for targeted re-sequencing studies because it allows

investigators to interrogate hundreds of genes at once with greatly simplified workflows and reduced consumption of rare research samples. At the forefront of this transformation is the Ion AmpliSeq™ technology, which enables the selective amplification of 10s to 1000s of target sequences in a single multiplexed PCR and meshes seamlessly with the Ion semiconductor sequencing platform. Here we describe features and applications of the Ion Ampliseq™ Comprehensive Cancer Panel (CCP), which provides ready-access to hundreds of genes, making it ideal for broad targeted re-sequencing studies aimed at understanding genetic variations in cancers. In this study, we applied the panel to the characterization of NCI-60 cell lines (MCF-7, MDA-MB-231, DU-145, SK-MEL- 28) derived from breast, prostate, and skin cancers. The CCP includes 61 genes that have been sequenced in one or more of the cell lines in previous studies using Sanger sequencing methods. Here, we confirmed the presence of previously reported mutations in these genes in the cell lines. For the 348 additional genes that were evaluated with CCP, we found previously unreported mutations, including missense and non-coding mutations, in several of the genes. Taken together, these findings not only confirmed the presence of known mutations in the NCI-60 cell lines from previous surveys, but also uncovered cell line-specific mutations in genes only evaluated by CCP. The ability to examine more than 400 high-profile genes at once empowers researchers to realize a more complete picture of the genetic variation that underlies different cancer types.

For Research Use Only. Not for Use in Diagnostic Procedures.

P004

Ready to Go for Next-Generation Sequencing: Extraction of Circulating microRNAs from Bovine Plasma

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Background - Analyzing circulating nucleic acids is a new and highly interesting field in recent science. In the year 2000, miRNAs were discovered in humans and it was only five years ago that cell-free miRNAs were found in plasma. Therefore, miRNAs in the circulation came into focus as a powerful tool for biomarker detection. Although there are several protocols for the extraction of circulating miRNAs with a following quantification of specific genes via qPCR, there is no method to extract circulating miRNAs from plasma samples and analyze them in a holistic way with Next-Generation Sequencing (NGS), the state-of-the-art technology in massive parallel sequencing that enables a comprehensive characterization of the whole transcriptome.

Methodology / Principal Findings - Here, a new method is presented to extract circulating microRNAs from bovine plasma. For the purification of total RNA, a modified protocol was applied for the miRNeasy Serum/Plasma Kit (Qiagen, Germany) that was used in combination with the QIAvac 24 Plus vacuum system (Qiagen, Germany). The 2100 Bioanalyzer (Agilent Technologies, Germany) reported high miRNA concentrations, which indicates the effective extraction of circulating miRNAs. The extracted RNA could directly be used for library preparation (NEBNext Multiplex Small RNA Library Prep Set for Illumina, New England BioLabs Inc., USA) and a subsequent small RNA-Seq analysis on the Illumina HiSeq2000 platform (Illumina Inc., USA). Library preparation was performed according to the manufacturers´ protocol with 35ng of RNA as starting material. A concluding High Sensitivity DNA Assay Bioanalyzer run revealed successfully amplified cDNA libraries with adapter-ligated constructs in the appropriate size that signifies the amplification of microRNAs. Raw NGS read data was analyzed with Genomatix software (Genomatix Software Inc., Germany).

Conclusions / Significance - The amount of RNA which was extracted by commercially available kits for circulating miRNAs from body fluids is only sufficient for qPCR analyses but not for the application of NGS, the standard sequencing technology of the future. We recommend the utilization of the presented extraction procedure for plasma samples when aiming for transcriptome sequencing (small RNA-Seq).

P005

The predicted secretome in the transcriptome of cereal cyst nematode, *Heterodera avenae*

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Objective: The cereal cyst nematode (CCN, *Heterodera avenae*) is a major pest of wheat (*Triticum spp*) crops in many countries. Cyst nematodes are obligate endoparasites that become sedentary within plant roots and induce the transformation of normal plant cells into elaborate feeding cites with the help of secreted proteins. These effectors are secreted as molecular tools to manipulate host plant cell cycle machinery to facilitate its infestation. Here we report the transcriptome analysis of *H. avenae* to identify putative effectors of parasitism.

Methodology: Herein, we have sequenced the transcriptome by Illumina GAIIx platform of pre-parasitic juvenile and parasitic female stage. Raw reads derived from *H. avenae* transcriptome were assembled using Abyss, Trinity and Velvet. Sequences were scanned for prediction of signal peptides and Subcellular localization with multiple algorithms SignalP 4.0, TargetP 1.1, Wolf PSort and SecretomeP2.0. Further functional annotation was performed with InterProScan and Blast2GO.

Results: We assembled 74 million Illumina paired end reads, generated 55,928 transcripts with N50 764bp and annotated 23,246 transcripts.23,246 contig were used for the open reading frame (ORF) prediction and predicted ORFs were undergone to secretome analysis which identified putative 728 sequences encoding secretory signal peptides. Sequences having transmembrane were excluded from analysis. Further, putative secretory proteins present in the extracellular region were used to identify orthologues in other plant parasitic nematodes. Gene ontology was assigned which suggested that several of the secreted proteins are involved in carbohydrate metabolism and proteolysis. Subsequently, few parasitism genes were found to be differentially expressed and validated using Quantitative RT-PCR. Additionally, development of the *Heterodera avenae* Transcriptome Database (HATDB) has been in progress with the view to provide most comprehensive information about the *H. avenae* transcriptome.

Conclusion: Here we report the *H. avenae* secretome and identified putative effectors of parasitism.Further exploration of these parasitism genes might reveal the weaknesses in the nematode life cycle that can be exploited to design novel nematode management strategies.

P006

Differences in microRNA composition over the course of lactation – Next-Generation-Sequencing of bovine milk and blood

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Small RNAs (< 200 nt), especially miRNAs, play an important role as regulators in all major physiological processes and can be found in most cells and biofluids. While they have been and still are studied heavily in humans, mice and some other organisms, comparatively little is known about their abundance in cows. To this date known mature miRNAs in humans still outnumber miRNAs in cows nearly 3 to 1. Additionally even less information is available about miRNA composition in bovine biofluids such as blood or milk and therefore whether milk

possesses its own defined small RNA profile differing from blood.

To generate a holistic overview of all present miRNAs in bovine blood and milk and to identify shifts in their miRNA profiles during the progressing lactation period, small RNA NGS was performed on whole blood and milk samples. Samples from 12 individual cows were taken in different stages of lactation (day 1, ~30, ~150 and ~250) to characterize miRNA composition during colostrum, early lactation defined by negative energy balance, peak lactation and late lactation 1-2 weeks prior dry off. RNA-Sequencing was performed using an Illumina HiSeq and subsequent data analysis was done independently using either the GGS and GGA stations from Genomatix Software GmbH (Munich, Germany) or using freely available python scripts and R-packages. Significantly regulation of miRNAs between different tissues and lactation stages was defined by a fold change of expression of at least +/-2 and a Benjamini-Hochberg adjusted p-value of less than 0.05. To validate these findings key miRNAs were quantified via RT-qPCR for fold change comparisons. Experimentally validated mRNA targets for regulated miRNAs were taken from the Tarbase 6.0 database from Diana Lab (Athens, Greece) and pathway analyses were generated using Genomatix Pathway System.

RNA-sequencing clearly showed that milk possesses its own unique miRNA profile compared to blood and highlights its changes during the lactation period. Sequence reads could be successfully mapped to 582 miRNAs in blood and 566 miRNAs in milk with 487 and 424 miRNAs respectively found throughout all time points. Differential analysis between milk and blood showed more than 200 significantly regulated miRNAs during each time point, clearly separating RNA composition of these tissues. While regulation shifts between different time points were scarce in blood, differential analysis in milk revealed significant changes during all time points with a clear emphasis on colostrum with 56 regulation changes between day 1 and ~30. Pathway analysis for affected targets revealed heavy influences on cell cycle progression, DNA repair, apoptosis and oncogenic defence indicating a potential role of miRNAs in milk not only for the mammary immune system but also as an active modulator of gene expression in newborn calves.

P007

Rapid Sequencing Of The Entire Human mtDNA Using Next-Generation Sequencing By Ion Torrent PGM

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Mitochondria are subcellular organelles that function as energy producers of the cells and regulators of the cellular metabolism. In humans mitochondrial DNA (mtDNA) is a 16,569 bp circular sequence and it is inherited only through the maternal lineage. Mutations in the mtDNA are associated with a range of human diseases and have also been implicated as a driving force behind the aging process. The main goal of our study was to design and describe reliable protocol for sequencing mtDNA with next-generation sequencing (NGS) which is now widely used in biological and medical studies.

We describe a method for rapid sequencing of the entire human mitochondrial genome by Personal Genome Machine (Ion Torrent PGM). With the aim of finding new disease-causing mutations, we sequenced 6 patient and 2 control mtDNA genomes. The method involves long-range PCR for specific amplification of the overlapping mtDNA loci, enzymatic shearing of amplified PCR products, barcoding of the samples, sequencing by PGM and quantitative mapping of sequence reads using the TorrentSuite software.

Based on the alignment to the reference sequence, we achieved an average read depth of 238x per individual sample (AQ20: 188x). Good coverage of the entire genome was also obtained: on average, 100x coverage was achieved for 87,3% of the bases and 20x coverage for 99,0% of the bases. The combination of high read depth and broad coverage of the genome enabled us to search for genetic variants with high confidence. The number of identified variants ranged from 8 to 38, where majority of them overlapped between the different samples, giving them even more reliable pathogenic value. To better assess their significance, all the variants were studied further using the MITOMAP human mitochondrial genome database. In the case of several identified variants, these could be linked to the observed patient's clinical profile.

The described protocol enables a simple and reliable identification of known and novel mitochondrial variants. It offers a fast and cost effective alternative to the classical Sanger sequencing protocols, and the ability to multiplex even larger numbers of samples by barcoding can further decrease the cost per sample. Another advantage is a straightforward data analysis, which does not require any advanced bioinformatics knowledge. Based on the readily achievable deep coverage, the method can also be used to identify heteroplasmic variants with higher resolution, which was much more difficult to study in the past.

P008

An Automated, High-Throughput Library Construction Protocol with Benefits for Low-Input Applications

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Innovation in Next-Generation Sequencing (NGS) has been focused on core sequencing technologies, with the optimization of library preparation playing a secondary role. We and others have previously reported on the advantages an engineered, high-fidelity polymerase for library amplification, which enables improved library quality and sequence coverage. To extend the benefits of low bias amplification, particularly to low-input applications, we recently developed a library construction protocol that incorporates the "with-bead" strategy conceptualized by The Broad Institute of MIT and Harvard. Together with the use of ultra-pure, high quality reagents for library construction, the "with-bead" protocol results in significantly higher recoveries of adapter-ligated molecules from lower amounts of input DNA. These benefits allow for fewer cycles of amplification, thereby further reducing the risk of PCR-induced bias, error and other artifacts that can affect library quality, sequence coverage and reliable library quantification.

We have developed an automated method for high-throughput library construction using the KAPA HTP Library Preparation Kit on the Biomek FX Laboratory Automation Workstation (Beckman Coulter). This method implements library construction from fragmented dsDNA to amplified library in one run, while supporting optional size selection and adapter indexing. By taking advantage of the flexibility and extendability of the Biomek software, several options are presented to the user in an intuitive, easy-to-use interface. For optimal results, the method employs unique automation components, designed specifically to handle small volumes of samples and precious, temperature-sensitive reagents. We will be presenting validation data for libraries constructed from different amounts of high-quality cell line DNA. Data on the use of the automated "with-bead" method vs manual library construction ("traditional" method) for library construction from precious FFPE samples for targeted capture, will also be included.

P009

Transcriptome Analysis on the Ion Proton™ System

Richard Fekete, Kelli Bramlett, Yongming Sun, Jeff Schageman, Luming Qu, Ross Hershorn, Charmaine San Jose Hinahon, Bob Setterquist

Life Technologies, United States of America

RNA-Seq technology has become widely utilized as a tool to understand the transcriptome of a given experimental system. This method utilizes next generation sequencing platforms to sequence a cDNA library in order to gain information about the RNA content and transcriptional status of a sample of interest. Profiling the transcriptome of a system in this way has become an invaluable tool in many genomic studies.

Ion Torrent semiconductor sequencing utilizes revolutionary technology, simplifying next generation sequencing instrumentation and greatly reducing sample to sequence workflow duration, making this technique more attractive for applications beyond basic research. The newly launched Proton™ system utilizes the same simplified chemistry first introduced with the Personal Genome Machine (PGM™). The increased sequencing depth of the Proton™ instrument and the Ion PI™ chip now lends this simplified sequencing technology to true whole transcriptome evaluation including sequencing analysis of polyadenylated RNAs, long non-coding RNAs, and non-adenylated transcripts.

Here we report transcriptome sequencing of ribosomal RNA (rRNA) depleted control RNAs analyzed on the Proton™ instrument with the Ion PI™ chip. The transcriptome profiles of two well-studied RNAs utilized in the historic Microarray Quality Consortium (MAQC) were analyzed by Proton™ sequencing. External RNA Control Consortium transcripts (ERCCs) were spiked into the control RNAs to provide a known set of RNA sequences useful in monitoring sample preparation, sequencing and data analysis. Differential expression profiles between Universal Human Reference RNA (UHRR) and Human Brain Reference RNA (HBRR) generated from transcriptome sequencing on the Proton™ compare well to profiles generated during the MAQC study. This study demonstrating over 50M mapped reads from each Proton™ transcriptome sequencing run, comparable gene expression profiles from three diverse analysis platforms, and good sensitivity and dynamic range as shown with the ERCC controls, solidifies the new Proton™ sequencing system as a viable platform for complex transcriptome analysis.

P010

Rapid whole genome sequencing investigation of a familial outbreak of *E. coli* O121:H19 with a sheep farm as the suspected source

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The availability of benchtop high-throughput sequencing instruments has made rapid whole genome sequencing of bacteria a potent tool in epidemiological investigations. In a recent case, verotoxin 2-positive *E. coli* O121:H19 was isolated from a Swedish patient and two asymptomatic family members. O121:H19 vtx2[+] was also isolated from sheep at a farm that the family had visited, and zoonotic spread was suspected. Current standard molecular typing methods were applied to the isolates (PFGE, MLVA), but were inconclusive with small degrees of profile variation both within the family and between the family and animal isolates. To resolve this, the genomes of the three outbreak isolates, a sheep isolate from the farm and two unrelated patient isolates were sequenced using Illumina MiSeq technology, with nanogram quantities of DNA from automated extraction protocols as starting material. Analysis of the generated data found single nucleotide polymorphism (SNP) variation strongly indicating that two of the outbreak isolates were of recent common origin, one of which was from the symptomatic patient. However, the third patient isolate as well as the sheep isolate were distinct from each other and from the isolate from the symptomatic patient. Thus, there was no evidence that the sheep farm was the source of the infection. Regions of interest were extracted from the generated sequences to produce backward compatible typing data such as verotoxin profile, MLST profile and the presence/absence/subtype of secondary EHEC virulence determinants. This analysis supported the PFGE and MLVA typing, indicating that all isolates in the outbreak investigation belonged to the same clone of O121:H19. The prevalence of this clone in ruminants and asymptomatic humans in Sweden should be investigated further. The emergence of quick and affordable lab methodology combined with standardized data analysis workflows will see WGS taking an increasingly important role in the routine work of veterinary and public health authorities in the next few years.

P011

AmpliSeq™ RNA: Targeted sequencing of genes on the PGM ™

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As Next Generation Sequencing matures, it is quickly moving into translational research applications where it has promise to be a useful tool for evaluation of human samples. RNA profiling using NGS (RNA-seq) is one of the applications where this potential is currently being realized. RNA-seq experiments have traditionally started with a whole-transcriptome library preparation that produces a sequencing template from all RNA species in a sample. However, in many cases, only a handful of the genes present are necessary to make a clinically relevant diagnosis.

We have demonstrated new technology that allows for RNA-seq from a panel of directed amplicons using an AmpliSeq™ approach with Ion Torrent semiconductor sequencing. This approach offers many advantages over microarray or qPCR such as faster turnaround and data analysis, sample multiplexing, lower RNA inputs, and ability to use degraded or FFPE-derived samples. In addition, the technique simultaneously provides quantitative gene expression information and gene sequence at the single nucleotide level.

We have compiled three gene panels for testing the method including a cancer panel, apoptosis panel, and a panel derived from the Micro Array Quality Control (MAQC) consortium studies. Starting with 10ng of total RNA, cDNA is made, followed by amplification using primers designed for targeted genes. Resulting amplicons are prepared for sequencing using the AmpliSeq™ technology and sequenced on the Ion Torrent PGM. We demonstrate that the technique produces results that are technically reproducible, quantitative, and have excellent correlation with qPCR using TaqMan® gene expression assays. Employing barcodes, we have also tested multiple samples on a single chip thereby increasing the cost-effectiveness of the tool for higher throughput laboratory settings.

P012

High Resolution HLA Typing of Blood Stem Cell Donors by Next Generation Sequencing

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Recent advances in Next-Generation Sequencing (NGS) technologies have enabled NGS as a proven alternative for classical Sanger sequencing for the characterization of the human leukocyte antigens (HLA). However, HLA typing is still a time-consuming, labor-intensive and expensive process with limitations to discriminate alleles at high-resolution level. Here, we describe an automated high-throughput workflow using the Hamilton Microlab STAR line of liquid handling systems and the Roche 454 GS FLX system for initial typing of blood stem cell donors. High quality DNA is extracted on a chemagic STAR liquid handling system using chemagen's nucleic acid purification system. On the second workstation, PCRs are automatically prepared in 384-well plates, and eight PCRs per sample are used to cover exon 2 and 3 of HLA-A and -B genes and exon 2 of HLA-DRB1 gene. PCR products are pooled and cleaned up with AMPure beads applying an automated protocol. After emulsion PCR, enriched beads are collected with the Roche 454 REM e system, integrated on the third workstation. In total, 380 samples are sequenced in one run on GS FLX system by multiplexing 96 samples and using 4 separated plate regions. Sequences are assigned with SeqHLA 454 software (JSI medical systems). In a cohort of 475 donors, clinically relevant ambiguities were observed only in HLA-B locus (in 1.3% of donors). In our experience, NGS allows a high resolution typing of HLA alleles in combination with high throughput of samples.

P013

MuA Transposase Enzyme Enables Fast And Easy DNA Library Preparation For NGS

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Thermo Fisher Scientific, Vantaa, Finland & Vilnius, Lithuania

Currently many different methods are used for Next Generation Sequencing (NGS) library preparation. Two major steps in the NGS library preparation workflow are the DNA fragmentation and the addition of adaptors for sequencing. While DNA can be fragmented using either enzymatic or physical shearing, the latter method requires additional equipment or has reproducibility issues. Following enzymatic or physical shearing, DNA fragments later in the workflow have their ends repaired and sequencing platform specific adaptors ligated. In contrast to conventional methods, library preparation using transposases can significantly simplify the process by combining the two steps into one. Transpososome treated DNA is cleaved into fragments containing uniform ends which are then easily modified to adapt the fragment library for various sequencing platforms. We demonstrate a library preparation protocol using the MuA transposase enzyme, which enables fast and easy NGS library preparation. In our workflow, MuA transposase enzyme simultaneously catalyzes fragmentation of double-stranded target DNA and tagging of the fragment ends with transposon DNA. A subsequent PCR step adds the platform-specific adaptors generating high yield of DNA fragments with the inserts ranging from 100 bp to 1000 bp.

P014

Next Generation Sequencing Sample Preparation Utilizing the Echo® Liquid Handler

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Labcyte, United States of America

The advent of Next-generation sequencing (NGS) has enabled researchers to overcome the limitations in resolution, scalability, and throughput experienced with capillary electrophoresis-based Sanger sequencing. While these technological advances have lowered the cost of sequencing, upstream library preparation remains a significant bottleneck and a prime target for automated liquid handling. The ability of Echo liquid handlers to acoustically transfer samples and reagents without tips or contact provides an efficient, contamination-free solution for genomic library preparation. The precision and accuracy of sub-microliter transfers from any microplate well to any microplate well accelerates and improves library pooling and normalization with less setup time in comparison to methods utilizing manual pipetting. In this work, the Echo 555 liquid handler was used to prepare libraries produced from E.coli for sequencing with the Illumina® MiSeq sequencer.

P015

Targeted Re-Sequencing Of 325 Inherited Disease- Associated Genes In A Family Trio And HapMap Populations Using The Ion Ampliseq™ Inherited Disease Panel And Ion PGM™ Semiconductor Sequencing

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The Ion AmpliSeq™ Inherited Disease Panel (IDP) provides ready-access to hundreds of genes involved in some of the most common inherited diseases in humans, making it ideal for broad targeted re-sequencing research studies of individuals and populations. The panel employs more than 10,000 primer pairs to amplify the coding exons of 325 genes that are associated with neuromuscular, cardiovascular, developmental, metabolic and other types of disorders. This panel is part of the transformative Ion AmpliSeqTM technology suite that enables the selective amplification of 10s to 1000s of target sequences in a single multiplexed PCR. The IDP is comprised of only 3 pools of primer pairs, requires just 30ng of sample DNA, and can generate comprehensive data from a sample on a single 316™chip using Ion PGM™ semi-conductor sequencing. In this study, the panel was used to examine the set of genes in a family trio from the CEPH population as well as in 24 unrelated HapMap samples from 4 ethnic populations (CEPH, YRI, CHB and JPN). We demonstrated Mendelian inheritance within the trio by analyzing known variants passed from parent to child. We also demonstrated highly similar performance of IDP with samples from all 4 ethnic populations tested as well as the ability to identify genetic variants between populations, which highlight the benefits of using the panel for studies of genetically diverse populations. Taken together, the results of this research study demonstrated that the IDP can be used to detect multiple variants in more than 300 genes in families and across populations and is ideally suited for a broad range of investigations, including genetic risk and susceptibility studies in families and disease-association research studies in ethnic populations. For Research Use Only. Not for Use in Diagnostic Procedures.

P016

Use of internal standard mixtures enables reliable NGS gene expression quantification at lower cost following multiple-round PCR and inter-gene convergence of transcript abundance

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Background: Next Generation Sequencing promises to enable cost-effective qualitative assessment of clinically important genetic variation as well as quantitative nucleic acid measurement of transcripts, breakpoint lesions and relative makeup of polymicrobial communities. However, there is a need to reduce quantitative sequencing costs and ensure inter-laboratory and inter-platform concordance. Discordance in quantitative sequencing data results, in part, from bias introduced during library preparation and platform-specific variation in sequence analysis (up to >1,000-fold variance). Cost of quantitative sequencing is primarily determined by method of library preparation and depth of sequencing required.

Approach: We addressed the challenges in quantitative sequencing library preparation through a novel combination of well-established methods, including: 1) massively multiplexed PCR with low primer concentration and touchdown thermal cycling conditions, 2) multiple rounds of PCR, and 3) inclusion of a standardized mixture of internal standards at known concentration. The massively multiplexed PCR under low primer concentration and touchdown thermal cycling conditions enables specific amplification of both high and low-abundance target amplicons to equimolar endpoint concentration. Because each nucleic acid target is at approximately equal abundance. at endpoint, this promises to markedly reduce the required read depth and thereby reduce quantitative sequencing costs by log-order magnitudes. However, without appropriate quality control the original quantitative relationships between nucleic acid templates will be lost during multiplex PCR-based library preparation. We solve this challenge through introduction of a standardized mixture of internal standards into each sample prior to PCR.

Results/Discussion: In preliminary studies, using multiplex PCR we bar-coded 333 unique target libraries from six equal aliquots of normal bronchial epithelial cell gDNA, each combined with a known concentration of serially titrated synthetic internal standard mixture (10^7-10^2copies). A 15x read coverage at each internal standard titration point provided a 10^8x equivalent quantitative sequencing depth even for genes in balance with the most dilute concentration of internal standard and preserved the original inter-gene copy number relationship. Based on these results we conclude that, compared to other commonly used quantitative sequencing library preparation methods, the novel approach reported here will reduce required read depth for quantitative sequencing by log-order magnitude. Further, when the same standardized mixture of internal standards is used, results will be concordant across different laboratories and platforms.

Molecular Diagnostics
P017 – P035

P017

Identification Of Distinct Subpopulations With Stem Cell Characteristics In Breast Cancer Types At Single Cell Level

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The cancer stem cell (CSC) hypothesis holds that cancers are organized in a hierarchical structure and derived from a cellular population with stem cell properties. CSC characteristics include self-renewal, tumorigenicity, multi-lineage differentiation as well as increased resistance to radio/chemotherapy-induced cell death. The CSC hypothesis implies that the re-populating cellular component of a cancer needs to be eliminated in order to achieve curative treatment. Current markers used to define progenitor and stem-like cells of breast cancers are highly controversial and may differ within various breast cancer subtypes. In the present work we aimed to study the heterogeneity of the progenitor/CSC component on a single cell level in different breast cancer subtypes.To this end individual cells were collected by flow cytometry, RNA was reverse transcribed, preamplified and the expression levels of multiple transcripts were assessed by qPCR. Through the use of gene expression analysis and single cell qPCR different sub-populations in estrogen receptor α (ERα) positive and ERα negative breast cancers have been identified. Moreover, we have elucidated a distinct molecular signature for CSC in these subtypes, indicating the existence of diverse previously unknown CSC/progenitor cell subpopulations. This data identifies possible pathways unique to CSCs and hence could be useful in identifying biomarkers and potential therapeutic targets to refine effective therapies.

P018

A multimarker qPCR platform for endometrial cancer biology characterisation

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Background - Endometrial cancer is the most frequent malignancy of the female genital tract. Despite such high prevalence, the understanding of its molecular background in terms of genesis, growth and progression still remains insufficient. The aim of this study was to characterise molecular basis of endometrial tumours through a quantitative PCR platform which encompasses potential molecular markers that might be of prognostic and predictive significance.

Methods - We have established a multimarker quantitative PCR platform to examine genes which might carry aberrations and thus be of significance in endometrial cancer biology. The study group consisted of 156 endometrial cancer patients, staged I-IV. Gene dosages of *TOP2A*, *ERBB1, ERBB2, ERBB3, ERBB4, c-myc, CCND1, ESR1, PI3K, RAD21* were determined in fresh-frozen tumour samples using SYBR-Green-based quantitative PCR assay, with *APP* and *3P* as reference genes. Measured values were analyzed with the use of qbasePLUS platform, Version 2.3. Inter-run calibration was implemented. Reference target stability was verified – values of M and CV were below 0.5 and 0.2, respectively. User-defined, separate for each gene, amplification efficiencies were assumed. Experimentally determined cut off values were used in aberration evaluation. All statistical analyses were performed using STATISTICA software, version 10. Gene aberrations were assessed (according to clinicopathological parameters) by Crosstabs statistics with Pearson's chi-square test. Statistical significance was assumed when $p<0.05$.

Results - Out of the studied genes ERBB family was found to be highly deregulated, together with its downstream effectors – *PI3K* and *c-myc*. Upregulated ERBB PI3K/Akt pathway was assumed whenever any of *ERRB* genes together with *PI3K* and/or *c-myc* were found amplified. ERBB family was upregulated in 54 (34.6%) cases. Entire ERBB PI3K/Akt pathway was upregulated in 30 (19.2%) cases. Activated pathway positively correlated with higher stage (p=0.0008), grade (p=0.001), histological typeII (p=0.0009) of the disease as well as with the presence of metastases (p=0.014).

Conclusions - Results obtained with the use of our multimarker platform demonstrate that aberrated gene dosage pattern of ERBB signaling network and PI3K/Akt pathway correlates with more aggressive clinicopathological features of endometrial cancer. The developed protocol yields coherent gene dosage measurements. This robust method might be used in clinical practice for more precise tumour characterization.

P019

Frequencies of CES1 Gene Polymorphisms In Patients With Metastatic Breast Cancer and Evaluation of Drug Response Effects in Capecitabine Users as Retrospectively

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Breast cancer is a frequently observed multifactorial disorder with the highest rate of mortality between women all over the world. Reproductive effects, hormonal effects, genetic effects and socio-economic situations are considered as factors that can cause breast cancer. The last stage of the breast cancer which is classified according to the stage of the existing tumor is metastasis. Lymph nodes, lungs, brain and bones are the most vulnerable regions to breast cancer metastasis. Medication takes an important part in treatment of metastatic breast cancer. Among these drugs, capecitabine is the most preferable drug due to its FDA approval and economic price. Capecitabine is absorbed in the intestines after converted to its active 5- fluorouracil compound by passing through several metabolic steps and exhibits cytotoxic effects in tumor cells. In one of the enzymes that is responsible for its metabolism is CES1, a carboxyesterase. It is considered that genetic alterations in the CES1 gene may cause different drug responses and toxic effects. For this reason, the aim of our study was first to analyse the frequencies of the CES1 I49V, S75N ve R199H gene polymorphisms that are located in exonic regions and trigger amino acid changes in the Turkish population and later the relation between these polymorphisms and the presence toxic side effects of capecitabine in cases with metestatic breast cancer. For CES1 I49V gene polymorphism, eight cases (13.3 %) had a wild type (I/I) and 52 cases (86.7 %) had a heterozygous(I/V) genotype from a total of sixty control group cases. From the total of twenty cases, five cases (25 %) had a wild type and 15 (75 %) had a heterozygous genotype. For CES1 S75N gene polymorphism, fourty-one cases (68.3 %) were found to carry a wild type (N/N), 16 cases (26.7 %) a heterozygous (N/S) and 3 cases (5 %) a homozygous genotype in control group. Whereas, in study group, 10 cases (50 %) were found to carry a wildtype (N/N), 9 cases (45 %) a heterozygous (N/S) and 1 case (5 %) a homozygous (S/S) genotype. All study and control groups cases (100 %) were found to carry the wild type (H/H) genotype for CES1 R199H gene polymorphism. When we compare the study and control group, an increase of heterozygous cases was observed in the study group, but no statistically significant difference was found. And also, no association was found between capecitabine-related drug toxicity and three CES1 gene polymorphisms that was investigated. This study is original, since it is the first one to investigate the frequencies of three CES1 gene polymorphisms and their association with metastatic breast cancer and capecitabine toxicity.

P020

Evaluation of a Microcapillary Electrophoresis (MCE)-Based Surveyor Scan KRAS Mutation Assay for the Pathology Routine

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Objectives: The introduction of novel monoclonal antibody colon cancer therapies (Erbitux, Vectibix) initiated the demand for accurate methods of gene mutation detection (KRAS, BRAF) in the pathology laboratory routine workflow. The industry responded with the development of IVD-CE marked assays tailored to the requirements of hospital environments. Depending on specimen throughput and available space open and closed kit assay systems on various molecular diagnostic platforms have been developed. In the current study we evaluate a microcapillary electrophoresis (MCE)-based SURVEYOR endonuclease KRAS mutation assay on the MCE WAVE instrument for its utility in a medium-sized pathology institute. As a quality measure we compare the novel MCE technology with the established real-time PCR and reverse strip hybridization assays in regard to test accuracy, ease of use and economic yield.

Methods: Histologically staged tumor tissues (154 colon, 6 melanomas, 1 thyroid cancer) were collected from the pathology routine with ethical patient consent. Genomic DNA was extracted from 3 x 10 mm thick FFPE sections with three different methods (DxS protocol, QIAamp®DNA FFPE Tissue Kit, cobas® DNA Sample Preparation Kit). DNA was quantitated with a Qubit® 1.0 fluorometer (Quant-iT™ dsDNA BR assay) and PCR reactions were set up on a Mastercycler ep Gradient S cycler. Mutation detection was performed with reverse hybridization strip assays (KRAS/BRAF and KRAS 12/13/61 StripAssays®, ViennaLab), the DxS TheraScreen® K-RAS Mutation Kit on a LightCycler 480 II system (Roche) and the SURVEYOR® Scan KRAS Mutation Detection Kit Exon 2 CE IVD with the WAVE® MCE System (Transgenomic, Inc.). Data were inspected visually and interpreted by the respective manufacturer´s software.

Results: Microcapillary electrophoresis of endonuclease-digested heteroduplex DNA amplicons performed over a wide range of input DNA with identical sensitivity and specificity as the widely used strip and real-time PCR assays. 65 out of 161 (40.4%) patient genomic DNA samples contained a mutation in the KRAS gene (Gly12Ala: 6, Gly12Asp: 20, Gly12Cys: 10, Gly12Ser: 2, Gly12Val: 17, Gly13Asp: 10). Data from the SURVEYOR® Scan corresponded 100% with 73 DxS TheraScreen and 88 strip assays with no additional mutations detected by the KRAS exon 2 gene scan assay.

Conclusions: Microcapillary electrophoresis is a reliable and fast high throughput tool for post-PCR mutation characterization. It can be used for a direct diagnostic patient assessment or as a cost effective screen for subsequent DNA sequencing. Additionally MCE supports quality control measures for the extracted DNA, the PCR efficacy, the efficiency of the nuclease digest and quantitative parameters for the analyzed DNA fragments. (**Supported by A. Menarini Diagnostics**)

P021

Development of a HRM-based detection method for DNA biomarkers in support of public health

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Biomarkers play a key role in public health because they are indicators of hazard, exposure, disease and population risk. They provide information for early detection, prediction, prevention, prognosis, diagnosis and response to therapy of diseases. Biomarkers can therefore be used to make group and individual risk assessments to support a pro-active public health policy.

There are different types of biomarkers. The most commonly used types of biomarkers are obtained from body fluids, of which blood is traditionally the most used although it requires a certain expertise for sampling and some target groups (e.g. young children), might be reluctant to providing samples. Urine might therefore be a valuable alternative as a source of biomarkers, because it is readily available and can be obtained by a non-invasive collection method, which is an advantage for large scale population studies. Until now, urine is mainly used as a source of protein biomarkers, although it also contains metabolites and nucleic acids. However, until now, urine-derived DNA has not

been used very often to measure DNA biomarkers.

DNA biomarkers are germline biomarkers (inherited) such as SNPs, deletions, insertions or other variations on the DNA sequence level. As genetic variation contributes to both disease susceptibility as to treatment response, the measurement of DNA biomarkers at large population level is key to the development of Public Health Genomics, where genome-based knowledge is used to benefit public health, by its integration into public policy and health services for the benefit of population health. This is eventually translated into more effective personalized preventive care and disease treatments with better specificity, targeted to the genetic makeup of each patient/person.

Different technologies exist to detect SNPs. Aiming at the development of a low-cost, fast and high-throughput method, applicable in large scale population studies; we envisaged the use of DNA extracted from urine, followed by a HRM-based detection of the respective SNP. The high-resolution-melting (HRM) technique is based on qPCR including a melting analysis, where the change in fluorescence caused by the release of a DNA-intercalating dye form the reaction mixture of dsDNA as it is progressively is heated, is accurately measured. Variations in the targeted DNA sequence will cause a difference in the dissociation (melting) curve, allowing detecting SNPs in the targeted fragment.

A comparison of six commercial DNA extraction kits has been performed to select the most appropriate kit allowing the extraction of high-quality DNA from urine samples. As a case study for the DNA biomarker measurement, we have chosen the SNP rs1051730 in the neuronal acetylcholine receptor subunit alpha-3 (CHRNA3) gene which is associated with cigarette smoking intensity and nicotine dependence. We compared the commercially available Taqman kit with an in-house developed HRM-based method to detect this SNP.

P022

Fast And Cheap Screening Method Of A Novel Mutation In GJB6 Protein, Associated With Non-Syndromic Hearing Loss

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Major causes of prelingual non-syndromic hearing loss are mutations either in protein beta-2 gene (GJB2) or another gene coding for protein beta-6 (GJB6). Battelino et al. (2012) described a novel mutation (Met203Val, A → T transition at nucleotide 607) in protein GJB6 using Sanger sequencing in one patient with mild progressive hearing loss.

Recently, a new patient with similar symptoms of non-syndromic hearing loss was identified and it was found positive for the same mutation with sequencing.

To perform broader screening of patient's relatives we devised a rapid PCR-based method. As a technique qPCR is ideally suited for analysis of single nucleotide polymorphisms (SNPs) and has been increasingly used for this purpose since the advent of qPCR and as whole genome sequences have become available.

The saliva samples from the relatives were collected with buccall swabs. The automatic isolation of DNA was made by MagMAX™ Express Magnetic Particle Processor. We developed a new instrument protocol for DNA isolation with the MagMax Multi-Sample DNA kit. The concentration of the samples was measured by *absorbance* and the amount was normalized to 10ng/μl. A SNP TaqMan assay targeting a novel mutation of the GJB6 gene was developed. The assay was evaluated on the Life Technologies ABI 7500 Fast Real-Time PCR system and the final analysis was made by the Genotyping Software.

We confirmed the novel mutation A → T in the patient's father's relatives. The mutation was confirmed in 8 samples (heterozygotes A/T) out of 18 tested. The mutation was specific for the father part of family tree and it was never found in genes of non-relative individuals, which were also included in the study.

The results of our screening study help us to define the samples which we will use for next generation sequencing using the Ion AmpliSeq™ Inherited Disease Panel for the Ion Torrent Personal Genome Machine sequencer. With this approach we aim to get a better understanding of the role of mutations in other genes in correlation with the GJB6 mutation described here.

P023

Effect of HPV-vaccination on HPV-prevalence in Belgium

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Objectives - Persistent infections with high-risk human papillomaviruses (HPV) are the mayor risk factor for occurrence of cervical neoplasm: 99% of the cervical cancers are associated with high-risk HPV infection. Since type 16 and 18 are responsible for 45% and 18%, respectively, of the cases in Europe, more and more countries including Belgium set up HPV-vaccine programs, protecting against types 16 and 18 in order to reduce the occurrence of cervix cancer.

This study aims to assess the HPV 16 and 18 states in Belgian women in 2012, determining the effect of HPV-vaccination 2 years after the onset of the program.

Methodology - Cervical smears of 1700 women between 18 and 64 years and resident of Flanders (North Belgian) were screened during a 12-month period. All samples were tested by qPCR, using the Cobas® 4800 HPV test. The test targets a sequence of 200 nucleotides within the polymorphic L1 region of the HPV genome of 14 high-risk HPV types identifying 3 subgroups, namely HPV 18, HPV 16 and "others"(31, 33, 35, 39, 45, 51, 52, 56, 58, 59,66 and 68) at clinically relevant detection levels.

Results - The prevalence of HPV 16 in the tested women was 4% with the highest prevalence in the group aged 25-35 years old, while HPV 18 was detected in only 1% of the cervical samples. Other HPV-types counted for 13% and showed the highest prevalence.

Conclusion - The current study implies that the HPV peak has shifted from the previously dominant high-risk HPV type 16 in cervical neoplasm, to the other high-risk HPV group. Overall, we may conclude that, taking previous European epidemiological surveys into account, trends in Belgian HPV landscape have changed, since the introduction of the HPV-vaccination program (**Arbyn et al. 2009, 2012).**

References - Arbyn et al. Prevaccination distribution of human papillomavirus types in women attending at cervical cancer screening in Belgium. Cancer Epidemiol Biomarkers and Prevention 2009;18: 321-330.

Arbyn et al. EUROGIN 2011 roadmap on prevention and treatment of HPV-related disease. International Journal of Cancer 2012. *In press.*

P024

Upstream Open Reading Frames Are Regulated By Nicotinic Acetylcholine Receptor Subunits Associated With Smoking And Smoking-Related Disorders

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Nicotine addiction poses a major health problem worldwide and is known to considerably increase the risk for diseases such as cancer and cardiovasculopathies. Nicotine both modulates nicotinic acetylcholine receptor (nAChR) subunit expression in various, mostly still unknown ways and acts as a receptor ligand. The genes coding for nAChRs are therefore suspected to play a key role concerning smoking behaviour and related disorders. Especially post-transcriptionally regulatory mechanisms are considered to be involved in the modulation of nAChR subunit expression by nicotine. Such mechanisms are often caused by cis acting sequence elements like internal ribosomal entry sites, microRNA-binding sites and upstream open reading frames (uORFs) located within the untranslated regions (UTR). Therefore, we performed a systematic search for functionally relevant uORFs in the 5'UTR of the nAChR genes *CHRNA3*, *CHRNA4* isoform 1 and 2, *CHRNA5*, *CHRNA7* and *CHRNB3* that are assumed to be linked to nicotine dependence and smoking-related diseases. Reporter gene assays revealed that *CHRNA4* isoform 1 and *CHRNA5* harbor functional uORFs that are able to significantly downregulate the protein expression of the subsequent gene. qPCR tests ruled out that the results of the luciferase assay were due to a transcriptional effect for both genes. Next, we tested by luciferase assay if the ATG start codon of the uORFs of *CHRNA4* isoform 1 and *CHRNA5* are able to initiate protein translation, which would be a basic requirement for the actual translation of the uORF itself. Regarding the uORF of *CHRNA4* isoform 1, our experiments suggest that the ATG of this uORF is recognized efficiently by the ribosomes to initiate protein translation. Concerning the uORF of *CHRNA5*, our findings imply that other mechanisms have to be taken into consideration, including stalling of ribosomes at the uORF or leaky scanning. Consequently, our study revealed that two of the major nAChR subunit genes contain functional uORFs that are capable of regulating gene expression.

P025

Quantitative Allele Spesfic Amplification (quasa) In Residual Disease Monitoring Of Hariy Cell Leukaemia

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Quantitative allele specific PCR amplification has a long history in mutation detection. It also has significant recognised weaknesses. A single bases mismatch at the 3'end of a primer is insufficiently to confer complete specificity of priming leading to false positive results. We have developed a tool kit of techniques that can be used to enhance the discrimination of the approach enabling complete sensitivity. This approach has been used to successfully monitor and quantify the BRAFV600E mutation as a residual disease marker in hairy cell leukaemia. The results were superior to the current gold standard of flow cytometry.

P026

LoopTag Real-Time PCR Probe System for Sensitive Pathogen Detection

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In routine laboratory diagnosis quantitative real-time polymerase chain reaction (qPCR) is a powerful method to detect micro-organisms in sample materials of different sources. We developed the patented LoopTag real-time PCR probe system [1] as a new qPCR technology for pathogen detection of isolated DNA from different sample materials (e.g. swabs, liquor cerebrospinalis, cultures, ticks). The LoopTag is a gene-specific bimolecular structure which forms during the hybridization of a labeled primer together with a labeled probe on the elongated strand. In detail one PCR primer carries 5' a short target-unspecific sequence which is labeled with an acceptor dye. After the primer elongation, the gene-specific LoopTag probe hybridizes to this strand. The probe carries a gene-specific and a short primer-complementary target-unspecific 3'-sequence which is labeled with a donor dye. This brings both dyes immediately in proximity and elicits a FRET signal that can be monitored during the amplification. Thus the design has an optimal FRET distance for a good signal-to-noise ratio. The FRET signal is proportional to the formed amount of PCR product. The LoopTag system was tested for detection of clinical relevant organisms. By using a complex-specific primer pair all members of the *M. tuberculosis* complex were detectable with a limit of 2 gene copies per sample. Detection of *Borrelia spp*. All members of the *Borrelia sensulato* complex can be amplified with a detection limit of 1 to 70 gene copies per sample with a *Borrelia sensulato* complex-specific primer pair. Most *Borrelia sensulato* species can be differentiated by melting curve analysis. Only using huge amounts of DNA (10^5-10^6 copies per PCR reaction, except with *B. hermsii*) false positive signals with nonpathogenic *Borrelia* strains and with *Treponema* strains are available. *Bordetella pertussis* or *Bordetella parapertussis* can be amplified with a detection limit of 10 gene copies per sample with a *B. pertussis* or *B. parapertussis*-specific primer pair. *Bordetella pertussis* can be differentiated from *Bordetella parapertussis* by melting curve analysis. The amplicon choosed, excludes the none-pathogenic *Bordetella holmesii* from detection. An internal standard (positive control) was always included into each PCR reaction to exclude PCR inhibition. LoopTag real-time PCR probe system is a new and reliable system for the real-time PCR detection and characterization of pathogens. It uses cheap conventional fluorescence dyes, exhibits an easy primer design, has no restrictions regarding the PCR product lengths and is highly specific. The LoopTag system is applicable for multiplex real time PCRs and melting curve analysis over a large temperature range.

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P027

Sensitive Methods for Detection of Secondary *KIT* Mutations in Gastrointestinal Stromal Tumours

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Aims - Gastrointestinal stromal tumours (GISTs) are the most common mesenchymal tumours of the digestive tract and are characterised by activating mutations of the *KIT* or the *PDGFRA* gene. Advanced GISTs harbouring a primary *KIT* mutation are treated with the tyrosine kinase inhibitor imatinib. During the course of treatment most patients experience disease progression and acquire resistance to imatinib. The most common resistance mechanism is the development of secondary *KIT* mutations in addition to the primary mutation. Until now it is not proven if

secondary *KIT* mutations develop during imatinib treatment or if rare cells with secondary *KIT* mutations are already present in the primary GISTs, called minor clones. This study aims to establish more sensitive methods than the conventional Sanger sequencing for detection of minor clones with secondary *KIT* mutations in primary GISTs with known secondary resistance.

Methods - A cohort of well characterised primary and secondary GISTs, diagnosed by experienced pathologists and with previously determined mutational status of *KIT*, was enclosed in this study. DNA was extracted from FFPE tissues by the BioRobot M48 (Qiagen) and quantified. For analysis the Roche 454 GS Junior FLX Titanium System was used. *KIT* exon 9, 11, 13, 14, and 17 target and sample specific primers were designed. A multiplex amplicon library was generated and used for emulsion PCR and parallel 454 sequencing. All steps were performed according to the manufacturer's instructions. Additionally a sensitive allele-specific PCR was established. Primers were designed for *KIT* secondary mutations. Amplification products were analysed by gel electrophoresis and the LightCycler® 480 System (Roche).

Results - The conditions for parallel 454 sequencing of *KIT* could be established in over 40 GISTs. In most samples *KIT* mutations previously determined by Sanger sequencing could be verified with 100-1600 reads per exon and sample. By this approach, we did not detect minor clones in primary GISTs. Therefore, we changed the experimental set-up to be able to gain more reads per exon and sample and thus to increase the sensitivity. Until now, we could not detect additional mutations while analysis is still ongoing.

Conclusions - Parallel 454 sequencing is an adequate platform to perform sensitive mutational analysis and is helpful for minor clone detection of secondary *KIT* mutations in GISTs. Whether it will be possible to detect minor clones prior to treatment still has to be determined. Allele-specific PCRs might be a good alternative for minor clone detection and is currently evaluated.

P028

Cancer Biomarker Research Using castPCR™ Technology

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Cancer biomarkers have applications in the diagnosis, staging, prognosis and monitoring of disease progression, as well as in the predication and monitoring of drug response. Profiling and validation research tools are needed that exhibit the combined features of high sensitivity and high specificity for cancers. However, the sensitivity of molecular methods such as DNA sequencing and conventional genotyping in tumor samples is limited, typically ranging from 2-20%. We have recently developed TaqMan® Mutation Detection Assays using our competitive allele specific TaqMan® PCR (castPCR™) technology for cancer biomarker research. TaqMan® Mutation Detection Assays were tested with >300 tumor research samples (either fresh/frozen or formalin-fixed, paraffin-embedded samples) and cell lines to assess mutation status at multiple independent laboratories. The results showed that castPCR™¢ technology can robustly detect mutations as low as 0.1% and has >99% concordance to other technologies including PCR-based technology and sequencing. In this study, a large panel of castPCR™ assays for AKT1, BRAF, CTNNB1, HRAS, KRAS, NRAS, PIK3CA, PTEN and TP53 genes were used for investigating somatic mutations in breast tumor research samples. Initially, 4 model FFPE cell lines were used to validate the assays. Mutant DNAs were titrated in the wild type DNAs from 50% to 0.1%. Mutations were identified down to 0.1% titration with high reproducibility. No false positives were found in non-tumor samples. The results obtained by TaqMan® Mutation Detection Assays for 20 breast tumor samples (FFPE/fresh frozen) were concordant to those reported by other methods. Our data showed that castPCR™ technology provides an excellent tool for ideentifying cancer biomarkers or confirming potential cancer markers such as those obtained by next-generation sequencing and other technologies.

P029

Validation of a real-time PCR method for SNPs analysis of known genes of lifestyle-related diseases among filipino adults

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Nutrigenomics is the study of the complex interplay between genes and food affecting health, blurring the boundaries between food and medicine, and heralding an era of personalized nutrition. Together with other chronic diseases such as diabetes mellitus, cancer and obesity, hypertension arise from family of diseases with complex and interrelated etiologies. Large-scale genome-wide association studies have presented genes associated with blood pressure and hypertension, where a significant association was found within SNP *ATP2B1* rs2681472, p value < 0.01, consistent both in Caucasian and non-Caucasian population groups. Among the current protocols being used in detection of SNP variation is the high resolution melting (HRM). Thus, in this study we evaluated an HRM-based method for SNP analysis of *ATP2B1* (rs2681472)using blood samples extracted from someFilipino respondents of the National Nutrition Survey.Specifically, the study aimed to establish method accuracy, inter and intra day precision, LOD and LOQ, PCR efficiency and linearity. Method accuracy was determined by comparing results generated from single pass DNA sequencing, a known gold standard for genotyping. Data obtained from sequencing conducted in Korea were concordant with data using HRM analysis, thus, implying method is accurate. Intra and inter-day precision of the method were found to be precise, supported by a very low %RSD of 0.11% and 0.14%, respectively. Instrument Detection Limit (IDL) was found at 30 cycles (in Cq value). Correlation coefficient using the method has met the acceptance criteria between 0.9-1.0. In conclusion, HRM was successfully applied for genotyping of SNP related to *ATP2B1* gene, particularly rs2681472. The method was found to be accurate and precise. It is recommended, however, that other validation parameters should be tested (ie. Method sensitivity, etc) in future studies.

P030

Cloning and Expression of Pakistani HCV NS3–4A Pprotease and Development of Robust in vitro Serine Protease Assay by FRET

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Hepatitis C virus (HCV) infection is a serious cause of chronic liver disease worldwide with more than 170 million infected individuals at risk of developing significant morbidity and mortality. Current interferon based therapies are suboptimal especially in patients infected with HCV genotype 3 (predominant genotype in Pakistan) and they are poorly tolerated, highlighting the requirement of new therapeutics. HCV non-structural protein-3 (NS3) protease and helicase domains are essential for viral replication; they are highly conserved among various HCV strains. In the current study, we enrolled 56 HCV infected patients from various regions of Pakistan and determined their genotypes, ALT level and virus titer by Quantitative PCR. We have cloned and sequenced NS3/NS4A from 4 of the HCV Serum samples by using nested PCR

approach and were able to express the viral region from Pakistan HCV 3a genotype. Nucleotide sequence alignment showed high level of identities among 3a genotypes. One of the samples (NCVI 01) showed unique amino acids substitutions, including R9Q, L332P, L354I, I605V and S622C. Three dimensional structures were determined and analyzed effect of substitutions on amino acids interactions. We further established fluorescence resonance energy transfer (FRET) based assays for detecting proteolytic activity of (NS3–4A) serine protease, using AnaSpec peptide, for high throughput screening (HTS) inhibitors against HCV. In future, this study could be of great interest in the development of HCV NS3 cell-based HTS FRET assay for genotype 3a and subsequent antiviral testing of drugs.

P031

The Regulatory Effect of 1, α, 25-dihydroxyvitamin D$_3$ on the Expression of Inflammatory Cytokines in Diabetic Retinopathy

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Background - Long term hyperglycemia leads to macro and micro vascular diseases. One of these microvascular diseases is diabetic retinopathy (DR) which results in blindness in patients during working age. Retina has highest O☐ uptake and glucose oxidation. Prolonged high glucose concentration provokes other alternative glucose pathways leading to accumulation of several metabolic end-products which induce oxidative stress in retina causing cellular changes of the expression of pro and inflammatory proteins. These pro and inflammatory proteins include the expression of interleukins 1-α, 1-β, 6,8,17,18 and 33 and their cognate receptors and other proteins such as tumor necrosis factor-α (TNF-α). Recent studies have shown that the active form of vitamin D 1, α-25(OH)2D3 , calcitriol, can protect pancreatic beta cells, improve insulin peripheral sensitivity, modulating immunologic response and protect cells from oxidative stress and apoptosis. Thus, this study aims to investigate the potential effect of calcitriol on immunological responses during the progression of DR and underlying antioxidant mechanism(s) during increased oxidative events in diabetes.

Method - ARPE-19 and 661W cell lines were treated with 750 µM H$_2$O$_2$, 5 and 25 mM glucose and 50nM calcitriol or normal cell culture media or 0.1% ethanol for 48 hours in standard cell line culture conditions.

Results - Data show that IL-33 was highly expressed in both cell lines treated with 5 mM glucose and down-expressed after 25 mM glucose treatment for 48h .The expression of IL-33 was increased after 750 µM H$_2$O$_2$ treatment for 48h. The expression of ST2 receptor was down-regulated after 5mM and up-regulated after 25 mM glucose and down-expressed after 750 µM H$_2$O$_2$ treatment for 48h compare to untreated cells for the same period of time. Expression of IL-33 and ST2 after treating ARPE-19 cells with 50 nM active D$_3$ and 5 and 25 mM glucose and 50 nM calcitriol has significantly increases the IL-33 and ST2 expression by 3 and 9 and 5 and 22 folds respectively compare to glucose treatment alone for 48h (P=0.0303; P= 0.0492 for IL-33: P=0.0433; P= 0.0229 for ST2).

Conclusion - The expression of IL-33 and its ST2 receptor in ARPE-19 and 661W after hydrogen peroxide and glucose treatment in vitro suggests a protective pathway of IL-33 during diabetic oxidative stress and a regulatory role of 1, α-25(OH)$_2$D$_3$ in oxidative stress during diabetic retinopathy through modulating inflammatory proteins expression with possible antioxidant effect.

P032

Tumour Targeting of Gene Expression Using Hyaluronic Acid-Polypropylenimine Dendrimer Conjugates

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Cancer gene therapy continues to evade widespread clinical application due to the paucity of effective gene delivery systems. Our work is concerned with the development of safe and effective gene delivery systems and such efficient gene delivery has recently been reported with the lower generation polypropylenimine dendrimers systems. Here we report for the first time on the successful use of HA to target gene expression to tumours *in vivo.*

Most non-viral gene delivery systems target gene expression to the lung on intravenous administration. With the aim of developing tumour-targeted therapeutics, a polypropylenimine dendrimer – hyaluronic acid conjugate (HA-DAB) was synthesized and confirmed. Hyaluronic acid moiety is known to target CD44 a cell surface receptor over expressed in some tumour cells while the polypropylenimine dendrimer facilitates gene transfer.

The HA-DAB conjugate formed nanoparticles in aqueous solution, presumably due to complexation of the amine and carboxylic acid group or chemical cross-linking during the deivatisation step. Complete DNA binding (DNA condensation) as shown by the limiting fluorescence of ethidium bromide occurred at a minimum polymer, DNA weight ratio of 1:1.

In vitro biocompatibility studies indicated that HA-DAB was more toxic than both HA and DAB alone. The *in vitro* transfection data mirrored this observation as less gene expression was observed with HA-DAB than DAB alone presumably because the toxicity of HA-DAB reduces the level of expressed protein actually observed. However HA was found to inhibit the gene expression observed with HA-DAB but not that observed with DAB, indicating that the gene expression observed with HA-DAB was in part mediated by internalization via the CD44 receptor. In vivo gene expression was targeted to the tumour with HA-DAB but not with exgen 500. DAB was found to be too toxic for *in vivo* use due to the colloidal instability of the formulation.

P033

Using a qPCR based method for screening several polymorphisms in COXI gene

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Introduction - Diagnosis and screening of polymorphisms in genes can be accomplished by various methods. Concurrent advance in molecular techniques,faster and more reliable procedures Be replaced.In this regard,in this research we used qPCR based method to rapid screening polymophism.

Methodology - We have combined Realtime PCR and ARMS PCR to Creation a rapid method for polymorphism screening.thus, we have designed two forward primer for each polymorphism locus . one of theme with greater affinity to locus that containing polymorphism nucleotide and another one designed to link to the same locus but with greater affinity to normal nucleotide.Primers were designed so that products of each one have a particular length. The difference in the products length of each primer evident by High Resolution Melt analysis. Presence or absence of polymorphisms and Approximate amount of them determined by analyzing the results of melt temperature.

Primers that designed for one locus is shown below: Forward primer for wild nucleotide : 5 ACGACCAC***A***TCTACAACG 3 Forward primer for polymorphic nucleotide : 5 TTCTAGGTAACGACCAC***G*** 3 Reverse primer : 5 TAGGAGAGAGGGAGGTAA 3 Highlighted Nucleotide is

Nucleotide that we want to screen.

This difference in length will cause the difference in melt temperature.It should be noted that annealing temperature of the primers selected so that even a single nucleotide difference cause the primer not connected.

Results - PCR results for all samples indicated expected difference between samples amplified with normal primers and samples amplified with polymorphic primers. Also, we measured the height difference in melt temperature analysis chart and by using them we were determined ratio of Polymorphic nucleotides to the normal nucleotides.

Conclusion - As regards that studied gene(COXI) is a Mitochondrial gene and that each cell contains many mitochondria genome the presence of polymorphisms in the mitochondrial genome is relative.Proportion of the genome containing polymorphic nucleotides was measured by height difference in melt temperature analysis chart. The key to success in this method are design of primers and exact determination of annealing temperature. In compliance with these cases, this method can be used to screen for the other Mitochondrial genes. And of course, with small changes in the data analysis this method also can be used for nuclear genes.

P034

qPCR Gene Expression Profiles in Peripheral Blood Mononuclear Cells of Breast Cancer Patients

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The aim of this study is to obtain gene expression profiles of peripheral mononuclear blood cells (PBMCs) of breast cancer patients. We performed gene expression profiling in the patients group ($n = 64$) compared with the group of healthy donors ($n = 20$). The patients group proportionally consisted of primary as well as metastatic breast cancer patients. For this purpose PBMCs were collected from peripheral blood using Ficoll Paque, followed with appropriate mRNA isolation and cDNA synthesis. The gene panel consisting of 45 breast cancer associated genes, previously established in TATAA Biocenter was assessed using 384 well qPCR platform Viia7. Data analysis was carried out using Genex Professional software and revealed 15 genes to be significantly differentially expressed in the patients group compared to healthy donors.

P035

New Composites Biomarkers for Colorectal Cancer Diagnosis

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Context: DNA methylation plays a key role in Colorectal Cancer (CRC) development by inducing a change of transcriptome profile to plausibly cause genomic instability in affected cells. Patterns of methylation are a very promising tool for screening and detecting cancer at different stages, since methylation tests can be performed using a stable material like DNA extracted from body fluids such as serum, stool and urine. While transcriptomic data and markers are largely published and made available, the same is lacking for methylation.

Method: with the goal of identifying a set of methylation-based biomarkers enabling non-invasive diagnosis of CRC, we developed a computational method consisting of: 1/collecting genes described in literature as hypermethylated (more than 2000 papers), 2/inferring their serum methylation from their tissue expression data available in GEO-NCBI, 3/perform a screening by cut-off values and 4/ test their resistance to noise on the cut-off.

Results: we isolated 95 published genes that we considered to be relevant for CRC association, so identifying a panel of genes with a strong diagnostic power when used in combination. A threshold of 5% above the maximum methylation value in the set of normals enables us to identify a set of 14 markers for the diagnosis of CRC. This affords discriminating CRC patients at high specificity (100%) and sensitivity (100%).

Conclusion: our findings suggest a relevant non-invasive markers set for the diagnosis of CRC and a strategy could be extended to identify other composites for the screening of other cancers.

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MALDI-TOF MS as confirmation tool for pathogens in drinking water.

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WLN is the center for water quality and water technology. WLN takes care for clean, healthy and tasty drinking water. WLN is progressive in the use of new and faster techniques. One of the newest techniques is the use of the Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) biotype. With this new technique WLN can identify indicators and pathogens such as *Escherichia coli*,*Enterococcus* and *Legionella* within a few minutes directly from their selective media. MALDI-TOF MS is based on the chemotaxonomy of microorganisms. A single colony of a target organism is put directly on a 96 target plate. After deposition the spots were overlaid with 1 µl matrix solution (2.5mg α-Cyano-4-hydroxycinnamic solved in 50% acetonitrile, 2.5% trifluoro acetic acid, 47.5% ultra-pure water). The matrix opens the cell wall. A laser irradiate the matrix sample, to divide it in little portions of peptides. The matrix evaporate and positive charged peptides become free. In the strong electric field the positive charged peptides are lined up. So these peptides have the same starting point, before they accelerate in the flight tube to get to their specific time-of-flight corresponding with their specific mass. Spectra are generate with the MALDI-TOF MS biotyper from Brüker Daltonik GmbH and compared with approximately 4000 spectra in the Brüker Daltonik GmbH database. In a log score 1 to 3 the MALDI-TOF biotyper defind the similarity of the known and unknown spectra. WLN has tested 67 reference strains and 316 samples, grown on their specific and general media. These microorganisms were confirmed with classic techniques and the MALDI-TOF MS biotyper. In table 1, you can see the conformity between NEN- and MALDI-TOF MS confirmation. The validation is performed according NEN-EN-ISO 16140. Legionella is divided in two stages, the genes Legionella scores 100% similarity. Legionella species has a lower score because 4 Legionella species are not present in the Bruker database. Certain peaks of the microorganism 'fingerprint' are conserved, the so called abundant proteins. These proteins are always there and make it possible to characterize microorganisms. Some microorganisms are not detected (Legionella species) by the Bruker database, because Bruker did not introduce these microorganisms. Therefore the Dutch water laboratories will generate a collective 'water database'.

P037

Quantification of Nitrifying and Manganese oxidizing bacteria with real-time QPCR to evaluate purification efficiency of sand filters in drinking water production

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WLN, Netherlands, The

Introduction - Dutch drinking water is the safest and cleanest drinking water of the world. Drinking water can be produced out of groundwater or out of surface water after extensive purification. Groundwater once felt down as rain/snow and trickled into the earth where it begun a journey that took decades. The earth is like a huge filter. On its way, groundwater takes lots of natural compounds from the earth, such as gasses, metals, ammonia etc. Often groundwater has manganese (Mn) and iron (Fe) concentrations above the WHO guidelines. Postextraction treatment in aerated sand filters is effective in decreasing Mn and Fe concentrations. In a aerated slow sand filter, biomass develops easy, which can decrease chemicals by biological metabolism.[2] Biological treatment of drinking water is more and more common.[1] *Leptothrix sp.* are known to be capable of oxidizing both iron(II) and manganese(II). *Leptothrix species* are sheathed filamentous bacteria that can generally be found in different types of aquatic environments. Ammonia is toxic in low concentrations for fish. Ammonia can be transformed in nitrite, and nitrite gives trouble to oxygen transport in the blood by baby's. Ammonia is also good feed for bacteria that causes biomass in the water distribution network that lead to higher risk of legionella. The transformation of ammonia into nitrite and finally into nitrate is a biological process called nitrification, caused by nitrifying bacteria, *Nitrosomonas* and *Nitrobacter* respectively. Nitrifying bacteria and *Leptothrix sp.* growth very slow, so a plate count method is not useful. Quantifying by QPCR is much faster.

Aim - We developed two QPCRs to test these bacteria to get a closer look on the efficiency of the sand filters. With the results we get we can optimize the nitrifying- and manganese oxidizing process for an even better quality of our Dutch drinking water.

Method - There is not much mentioned about *Leptothrix* in literature, We found a few primers[1]. One primer also hybridizes to *Sphaerotilus natans* a bacterium that is incapable to oxidize manganese but very similar to *Leptothrix.* Therefore we developed a Cy5-labeled probe that was highly specific for only *Leptothrix sp.* Nitrifying bacteria also called ammonia oxidizing bacteria (AOB). In literature we found primers corresponding with the ammonia monooxygenase gene (amoA-gen)[3]. For the real-time PCR we developed a FAM-labeled probe. To quantify these bacteria we need a standard curve. We made a plasmid with the amoA gene and the 16S rRNA of *Leptohrix sp.* The plasmid had a begin concentration of 101.000 cDNA/L, that is decimal diluted to approximately 10cDNA/L.

Conclusion - Our results showed us that biological treatment of groundwater is a good way to remove unwanted compounds. The start of natural biological removal takes long time, it would be interesting to see if we can fasten biological removal by adding *Leptothrix* and *Nitrosomonas*.

P038

Application of a Multiplex Real-time PCR Approach for the Quantification of Beef and Pork in Minced Meat and other Meat Products

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Meat remains an important part of the staple diet of a large proportion of the human population. Although sometimes sold as pure fractions, most meat products are often heterogeneous and are composed of meat of several species. Herein lies an important task of food control agencies, namely the verification of declared meat components. In this work, we report on a newly developed triplex real-time PCR approach for the quantification of meat fractions in different matrices. The method was developed for the quantitative analysis of beef and pork fractions in minced meat products, and was successfully extended to other meat products like salami and sausages. Beef and pork fractions are quantified over dedicated primer and probe sequences that specifically recognize the cyclic guanosine monophosphate-phosphodiesterase gene (cow) and the beta-actin gene (pig) respectively, against the backdrop of a universal sequence commonly found in mammals, namely the housekeeping gene myostatin. The presented method was validated on 50 commercially available minced meat products, randomly selected by the official food surveillance authority, and 30 other meat products with varying matrices. The limit of detection of the beef and pork specific triplex real-time PCR was 20 genome copy equivalents for both target genes. The measurement of uncertainty of the method was determined to be 1.99%. The validated method performed well in terms of handling, reproducibility and robustness and can be readily implemented for routine use in food control agencies.

P039

Study of Bacterial Diversity in the Topsoil and in the Hardpan in an Agricultural Soil by Metagenomics Following by Two Analysis Pipelines

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On earth, Bacteria are ubiquitous and even present in extreme environments (pH, temperature,…). In soils in particular, bacteria are very abundant (up to 10^9 cells per gram of soil) but still poorly characterized. Thus, it is of paramount importance to use relevant study and analysis procedures to ensure that the results obtained closely reflect the real-life conditions. In the present work, we analyze the bacterial diversity in the topsoil and in the hardpan in an agricultural soil using the metagenomics approach, with the Ion Torrent PGM sequencer. The soil samples was collected at three depths : 10 cm (topsoil), 25 cm (topsoil above the hardpan) and 45 cm (in the hardpan), in a tilled and an untilled plot. The taxonomic analysis of the reads obtained are carried out according to two different procedures with the RDP classifier program and with a confidence score threshold of 0 and 0.99. The 0 threshold is used to assign a species to all reads, each read being therefore assigned to its most closest known species. The threshold of 0.99 enables us to focus on reads being assigned to a species with a high degree of confidence. In this case, each read is assigned to the most specific rank having a confidence score higher than 0.99. The bacterial diversity was then compared between the different conditions. Results obtained demonstrate that the bacterial communities were not the same in the two horizons. For example, some classes of *Acidobacteria* were up to 11 fold more numerous in topsoil while others was until 12 fold more represented in the hardpan. The biomass and the bacterial diversity (Shannon index) were also greatly different between the two depths.

P040

Development of HRM assay for differentiation of *Raspberry bushy dwarf virus* (RBDV) coat protein sequence variants

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Raspberry bushy dwarf virus (RBDV) infects red and black raspberry worldwide. It is one of the causal agents of crumbly fruit disease, which is the most important consequence of the infection. It naturaly infects *Rubus* spp. and grapevine, but similar virus was found also in citrus. Several red raspberry and grapevine plants were tested for RBDV infection and coat protien (CP) gene of positive ones was sequenced. Consistent differences were found between grapevine and red raspberry sequences of CP gene.

The aim of the study was to develop and test several primer sets for High Resolution Melting (HRM) analysis and find *a simple solution for identification and differentiation of* grapevine and red raspberry RBDV genotypes. In previous study the plasmids with different CP inserts were produced and sequenced. Some of them were used for testing the newly developed assays. The primer sets for HRM were designed on the areas with two to five nucleotide differences in the nucleotide sequences. The expected qPCR products were 96 to 106 bp long. Adapted real-time PCR protocol was run on ABI 7500 Fast SDS (Life Technologies), and followed by dissociation protocol. HRM analysis was done using HRM Software v.2.0. and the best primer pair for differentiation of grapevine and red raspberry RBDV variants was selected. We demonstrated the usefulness of new assay for differentiation of two RBDV variants using HRM analysis. The assay will be tested on cDNA of known samples and evaluated for RBDV genotyping of field samples.

P041

Development of a multiplex qPCR assay with propidium monoazide for quantifying viable *Bifidobacterium animalis* and*Lactococcus lactis* in faeces.

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Yoghurts and fermented milk products represent real ecosystems where several bacterial species coexist and are involved in the product manufacturing. Determining the concentration of bacteria of interest in marketed products is particularly necessary to ensure that dairy products contain a known quantity of live bacteria per gram of product until the use-by date. Methods such as classical counting cultures on Petri dishes or new alternative methods like flow cytometry are usually used to quantify these lactic acid bacteria in finished products. Some bacterial species considered as health promoting bacteria are also used in fermented milk products and are expected to exert positive effects in gastrointestinal tract, after product ingestion. Health promoting bacteria have beneficial effects in the live and/or dead state; it is therefore important to be able to discriminate viable cells from dead cells. The quantification of these strains in faeces samples represents a way to estimate its concentration after passing through the digestive tract. Faeces are complex matrices where live more than 400 different bacterial species; thus, previous quantification methods (ie Petri dishes…) are usually not enough specific to quantify these strains of interest. It is therefore essential to establish a specific method being able to identify them and measure their numbers, and to differentiate viable from dead bacteria in faeces samples. In our work, we developed a method for quantifying viable cells of two strains of *Bifidobacterium lactis* and *Lactococcus lactis* respectively, in faeces by a multiplex PCR- based method, both strains being used together in fermented milk products. The procedure was based on the combined use of the DNA-intercalating agent propidium monoazide (PMA) and qPCR, using strain-specific primers and probes. Samples treated with PMA before qPCR results in selective amplification of DNA from viable cells with intact membranes. This technique could be a promising analytical strategy for selective quantification of specific viable cells in matrices like faeces containing high endogenous microbial community.

P042

Influence of sample particle size on subsequent steps in Real Time PCR GMO analysis

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Preparation of laboratory sample is a first and very sensitive step in GMO analysis.The aim of the present study is to demonstrate correlation of different sample particle size with DNA quantity and quality. Repeatability of the results obtained from the two GMO Real Time PCR quantification was shown. This is especially important because threshold level for GMO contamination is 0,9% in accordance with the Regulation (EC) No. 1829/2003 and Serbian GMO law since 2009.

For that purpose, a control laboratory sample of 1% GM positive soybean seeds were grinded on the laboratory mill within different time spans in order to achieve different particle sizes. Six samples with different particle size were prepared: 850-1000 µm; 250-425 µm; 200-250 µm; <150 µm; <75 µm; <45 µm. DNA isolations were performed in duplicate and DNA quantity and quality were estimated by UV spectrophotometry. GMO quantification was done by Real Time PCR analysis in accordance with JRC method: "Event specific method for the quantification of soybean line 40-3-2 using Real Time PCR". The concentration of isolated DNA was increased with decreasing particle size of the sample. In rough grinded sample DNA concentration was 9,2 ng/µl while in the finest milled sample <45 µm was 100 ng/µl. In addition, absolute difference of estimated GMO content between two parallel probes was changed. In hardly grinded samples difference in GMO concentrations were up to 1% of absolute value, unlike in samples with finest granulation, were distinction were negligible.

Heterogeneity in sample particle sizes distribution influences amplification efficiency taxon specific soybean genes(lectins) and transgenes (Roundup ready gts 40-30-2), resulting in significant impact on Cq value. This consequently leads to under- or over-estimation of GMO content.

P043

Detection and Quantification of Sulfate Reducing and Sulfur Oxidizing Bacteria in Corroded Wastewater Systems using Quantitative Real-Time PCR Technique

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Biogenic sulfuric acid corrosion (BSA) is one of the most serious and costly problems affecting the world´s sewerage infrastructure (e.g. concrete sewer pipes) and wastewater treatment (e.g. digesters). A complex microbial ecosystem, comprising sulfate reducing and sulfur oxidizing bacteria (SRB and SOB, respectively), is involved in the BSA process. The bacterial activity in the wastewater systems creates a sulfur cycle which can lead to bacterial formation of sulfuric acid (H_2SO_4) and consequently to corrosion of concrete. 20% of the total damage of concrete structures in sewer systems seems to be caused by BSA leading to global repair costs of several billions of dollars per year.

Besides optical checks and acid analysis, currently, no precise test procedure is available for the detection of BSA attacks. Therefore, the aim of this research project (funded by Bundesministerium für Wirtschaft und Technologie, Zentrales Innovationsprogramm Mittelstand, ZIM-Kooperationsprojekt) is the development of a standardized biochemical test system for the accurate determination of the BSA potential in wastewater systems, especially in digesters.

This test system will include (i) detection and quantification of SRB and SOB in the digested sludge and biofilm growing on the concrete surface, respectively and (ii) determination of the concrete corrosion potential carried out in specific simulation chambers inoculated with SRB and SOB and concrete specimens. A combination of different conventional microbiological as well as molecular-biological techniques such as polymerase-chain-reaction combined with denaturing gradient gel electrophoresis (PCR-DGGE), sequencing and phylogenetic sequence analysis and fluorescence *in situ* hybridization (FISH) will be applied to detect and identify the relevant SRB and SOB involved in the BSA process. On the basis of these results specific primer systems for quantitative real-time PCR (qPCR) will be selected from literature data or as needed newly designed, and qPCR with these primer set ups will be evaluated. Generally, by means of qPCR correlations between numbers and types of sulfur-related organisms and the probability or rate of corrosion shall be investigated. As a result, quantified information shall be gained about the extent of damage so that further evaluations about the structure stability can be made.

The test procedure would be a novel method to identify a BSA attack by quantitative data of the involved SRB and SOB populations correlated to corrosion potential in wastewater systems. It would have an enormous economic impact worldwide, since it would provide the opportunity to discover BSA sufficiently early thusly preventing expensive modernization costs.

P044
Insights into the Microbiota of the Bovine Uterus

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Recent years' advance in sequencing technology has resulted in extensive new knowledge of the microbial ecology of different environments. We used the technology to investigate the causality of endometritis, which is an inflammation in the inner lining of the uterus affecting up to 20% of dairy cows in Denmark following calving. Endometritis is linked to reduced reproductive performance, which is costly for the farmer. With incomplete knowledge of the bacteria involved, treatment is performed without an option for choosing the best suited antimicrobial agent, which may lead to unnecessary antibiotic resistance development. Slaughterhouse samples were analysed in order to obtain information on the uterine microbiota from both cows with endometritis and cows without signs of endometritis from a variety of herds.

We sampled uteri from cows (n=50) from a slaughterhouse in Holstebro, Denmark. An incision was made into the right uterine horn and an endometrial biopsy was taken with a pair of sterilised scissors. The endometrial surface was sampled with a cotton-swab through the same incision. All samples were immediately put in RNAlater. The DNA was extracted with the Maxwell 16 LEV Blood kit (Promega), the 16S rRNA PCR was performed with primers targeting the V2 region, and the 454 next generation sequencing was performed by GATC.

Previous results have shown that *Proteobacteria* and *Tenericutes* are the most important bacteria phyla in the uterus of healthy cows (Machado *et al*. 2012 and Galvão *et al*. 2012) while *Escherichia coli, Trueperella Pyogenes, Prevotella* species and *Fusobacterium necrophorum* have commonly been associated with endometritis (Sheldon 2006). Preliminary results indicate that we often find bacteria from phylum *Actinobacteria*in the healthy cows and often bacteria from phyla *Bacteroidetes* and *Fusobacteria* in cows with endometritis.

P045
Quantification of infectious pathogenic Campylobacter species in water using QPCR and PMA.

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Ingestion of Campylobacter via food or water is one of the main causes of acute diarrheal disease. Campylobacteriosis is normally a self-limiting disease, but complications like reactive arthritis or Guillain-Barre syndrome can occur. Especially birds and rodents are hosts of Campylobacter which are introduced in the drinking water pathway. Contamination of surface water is caused by excretion of campylobacter in the feces of these animals. To prevent infection with campylobacter via drinking water these bacteria have to be removed during purification of surface water meant for drinking water. A good and reliable method for detecting infectious Campylobacter species is very valuable in the Netherlands for the reason that there are now residual disinfectants used in drinking water to kill pathogenic bacteria. And therefore it is essential to know that during purification of surface water Campylobacter is diminished to acceptable levels to fulfill the legislation and to protect the consumer for disease. In this study we developed a quantitative real-time PCR for infectious pathogenic Campylobacter. To achieve this we combined the PCR with a Propidiummonoazide treatment (PMA) to detect just intact cells which can be infective. The method is compared to a classical most probable number culturing Technique and showed a good correlation between dead and live Campylobacter. The Validation is according to ISO 16140 in which Repeatability, Reproducibility, accuracy, detection limit and uncertainty were determined. Our conclusion thus far is that the QPCR for Campylobacter in water can be used in routine; more experience has to build up with more real samples to understand the effect and usefulness of PMA treatment in comparison to infectious behavior of campylobacter and the effect of water treatment processes as UV-disinfection.

P046
Selection and validation of reference genes for quantitative gene expression studies by real-time PCR in brinjal (*Solanum melongena* L)

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Objective: Analysis of gene expression patterns leads to functional understanding of biological processes. Quantitative real-time PCR has become the most commonly used technique for in-depth studies of gene expression. In order to quantify gene expression variation of specific mRNA, accurate and reliable normalization of one or more suitable reference genes must be selected to compare the target mRNA transcripts across different samples and tissues. In the present work, we illustrate the first evaluation of potential internal control genes across different developmental stages for reliable quantification of transcripts by real-time PCR.

Methodology: Seeds of egg plant (CV. Pusa Purple Long) were procured from National Seeds Corporation Limited, Indian Agricultural Research Institute (IARI), New Delhi. India Seeds were germinated and plants were grown under controlled conditions. Young leaves, stems and roots were collected for analysis at 20 days while, flower buds, open flowers and mature leaves were collected after 45-50 days.RNA was isolated and cDNA was prepared. Homologous housekeeping gene sequences were identified from Solanaceae family from NCBI, cloned and sequenced.Quantitative Real-time (qRT-PCR) was performed using SYBR Green I technology in Realplex^2 thermal cycler (Eppendorf). Data was normalized by using three different statistical softwares; BestKeeper, NormFinder and geNorm for measurement of the stability of

expression.

Results: We have evaluated the expression stability of six candidate reference genes (*18S rRNA, aprt, GAPDH, cyclophilin, actin*, and *RuBP*) in a set of six samples representing tissues of egg plant at different developmental stages. The candidate genes were cloned from cDNA and analysed by real-time PCR. The expression data was further analysed by three statistical methods (geNorm, NormFinder and BestKeeper) which identified *18SrRNA, cyclophilin* and *aprt* as the most stable and suitable reference genes in egg plant. This was further confirmed in four different varieties and two representative lines of transgenic egg plant.

Conclusion: In conclusion, *18SrRNA, cyclophilin* and *aprt* could be used for normalization of real-time PCR data for gene expression studies in egg plant.

P047

Preliminary Study For Screening Of Potentially Pathogenic Bacteria In Poultry Meat

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Poultry meat is one of the most widely available meats all over the world. The widespread use requires specific food control systems, with the objectives of producing safer food and reducing the outbreaks of foodborne illnesses. Despite efforts to improve hygiene and meat handling procedures, food remains one of the most frequent sources of infection in the world. While numerous potential vehicles of transmission exist, commercial poultry meat has been identified as one of the most important food vehicles for harmful organisms. Diagnosis and detection of pathogenic bacteria is commonly based on classical culturing and serological identification methods, which are laborious and time-consuming. In many places this prevents routine implementation for screening of meats intended for human consumption. The objectives of our study were to design an inexpensive, high-throughput, reliable workflow for rapid, highly sensitive and specific detection and identification of contamination of a poultry meat samples with potential harmful organisms (*Salmonella enterocolitis*, *Campylobacter jejuni*, *Campylobacter coli* and *Listeria monocytogenes*). Poultry meat samples were collected from several different sources. The automatic isolation of DNA was performed by MagMAX™ Express Magnetic Particle Processor with the MagMax Multi-Sample DNA kit. The concentration of the samples was measured by *absorbance*. Already published assays targeting specific genes of *C. jejuni*, *C. coli*, *L. monocytogenes* and *S. enterolitica* were used for final detection using the Life Technologies ABI 7500 Fast Real-Time PCR system. The evaluation of the results of our study shows that the designed workflow meets the objectives required for broad implementation, as it is inexpensive, high-throughput, fast, reliable and easily automated. Furthermore, our preliminary results, together with the results of several other studies, still raise concerns about the safety of poultry meat. Together with campaigns aimed towards raising the public awareness about the importance of adequate heat treatment, implementation of inexpensive screening of the pathogens, which are most commonly found in poultry meat, should lead towards considerable decrease of the risk for human health in the future.

P048

Regulation of HIF-1alpha and vasohibins during follicle maturation and corpus luteum formation and function in bovine ovary

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The aim of this study was to characterize expression patterns of hypoxia inducible factor-1alpha (HIF1a) and Vasohibin family members in bovine ovary to further enlighten their interaction during different stages of ovarian function. This includes follicular maturation until ovulation, corpus luteum (CL) formation, its function during luteal phase and its regression. Experiment 1: antral follicle classification occurred by follicle size and oestradiol-17beta concentration in the follicular fluid (FF) into 5 groups (<0.5, 0.5-5, 5-40, 40-180 and >180 ng/ml). Granulosa cells and theca interna 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, 3-4, 6-7, >8). Expression of mRNA was measured by RT-qPCR and concentrations of hormones in FF by ELISA. The results obtained showed a remarkable inverse and significantly regulated expression pattern in both follicle and CL. While vasohibin-1 and vasohibin-2 factors decreased, HIF1a increased significantly along with follicle maturation. During formation, function and regression of CL, HIF1a decreased while vasohibins (1 and 2) remained up regulated. These results could lead to the assumption that the examined factors are involved in the local mechanisms regulating angiogenesis and that the interaction between proangiogenic (HIF1a) and antiangiogenic (Vasohibin-1 and Vasohibin-2) factors impacts all stages of bovine ovary function, namely during follicle maturation and growth and during CL formation, function and regression.

P049

Quantitative Analysis of Transcripts of the Open Reading Frames of *Sugarcane Yellow Leaf Virus* Genome by One-multiplex RT-PCR

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The one-step multiplex RT-PCR was used for the simultaneous detection and quantification of ORF0-1, ORF2, ORF3-4 and ORF5 of Sugarcane yellow leaf virus (SCYLV) in sugarcane tissues. The GeXP multiplex RT-PCR assay system allows the detection of low-abundance gene transcripts, for example it detected as few as 20 copies of varicella zoster virus gene-specific transcripts. Amplification products were resolved by capillary gel electrophoresis and detected by fluorescence spectrophotometry. Sink leaves had higher transcript levels of ORF0-1, which codes for a suppressor protein, than source leaves and internodes. The transcript levels for ORF3-4, which code for capsid protein and movement protein, were significantly lower in source leaves in two of the three cultivars than in sink leaves and internodes. The differences between the cultivars could not be related to SCYLV-resistance, however. Obviously the transcription of the viral open reading frames, especially of the gene for the suppressor protein, is modulated in the plant.

P050

Molecular characterization and phylogenetic relationships of newly discovered Potato virus X (PVX) strain from Peru

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Potato virus X (PVX), a type member of the genus *Potexvirus* (*Alphaflexiviridae*), is one of the most widespread potato viruses, infecting a

wide range of hosts, mostly within *Solanaceae* family. It was shown to induce severe symptoms and dramatic decrease of yield, when occurring in co-infection with some potyviruses, especially Potato virus Y.

We employ the strategy of Illumina deep sequencing of small RNAs to obtain complete or near complete genome sequence of PVX from 5 symptomatically infected greenhouse (*Solanum tuberosum* and *Datura* sp.) and 3 field samples (*Solanum tuberosum*), collected in Peru. In analysis step, the combination of de-novo short reads assembly (Velvet) and reference reads mapping (MAQ) was used to generate the genome sequences of PVX. Several primer pairs were designed to allow for an efficient complementation and validation of obtained genome sequences using Sanger sequencing. Alignment and phylogenetic analyses were performed using MEGA 4 and SplitsTree 4.12.3 including all complete genome sequences of PVX available from NCBI GenBank database. Based on the alignment, RT-PCR primers were also designed, allowing efficient discrimination between different PVX strains detected in this study.

The phylogenetic analyses assigned PVX samples into three different groups of isolates. (1) Two field-collected samples cluster with other known South American isolates. (2) Five samples from greenhouse infection represent new strain of PVX with high level of sequence dissimilarity to other sequenced isolates (~16%). The new PVX strain was also detected in a field collected sample of potato, which was infected with the mixture of two PVX strains - the new strain and (3) a strain from Eurasian group. Our study shows the utility of small RNAs deep sequencing for successful viral strain differentiation and discovery of new viral strains. Moreover, the discovery of previously unknown PVX strain in Peru could indicate existence of higher diversity of this potato pathogen in the region, which would be in agreement with the high diversity of cultivated and wild potatoes present there. A similar pattern may be expected also for other potato pathogens.

P051

Mammary immunity of different cattle breeds measured with microfluidic high-throughput RT-qPCR

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The mammary immune system of cows and especially the immune response to mastitis (inflammation of the udder) is often studied in cultured primary bovine mammary epithelial cells (pbMEC) *in vitro*. To find possible genetic mechanisms responsible for a varying phenotypic susceptibility to mastitis in modern and ancient cattle breeds, pbMEC of the modern breeds Brown Swiss and Red Holstein as well from the ancient breeds White Park and Highland were non-invasively cultivated from milk and stimulated with heat-inactivated mastitis pathogens. A microfluidic high-throughput RT-qPCR platform was used to analyse the relative mRNA expression of 45 genes belonging to the innate immune system (complement cascade, inflammatory cytokines, chemokines, antimicrobial peptides, acute phase proteins, TLR-signalling, inflammasome, scavenger receptors, reference genes and others). Additionally, three proteins in the cell protein extract were measured via ELISA. A fast and strong reaction to *Escherichia coli* and a weak response to *Staphylococcus aureus (S. aureus)* was observed, which is in accordance with many other studies. Mostly inflammatory cytokines and chemokines along with antimicrobial peptides and the acute phase protein Serum Amyloid A3 were activated. Breed differences ($p<0.05$) were detected in the basal (unstimulated) expression of 16 genes. In the modern breeds, more genes were regulated ($p<0.05$) by the treatments. Expression of 8 genes differed between breeds ($p<0.05$) after stimulation with the pathogens. A Principal Component Analysis showed a clustering of the breeds in the basal gene expression, but not after stimulation. Lactoferrin and Serum Amyloid A protein levels showed breed differences ($p<0.05$) in control and *S. aureus* treated cultures. Overall, the ancient breeds seemed to have higher basal immune levels and a less pronounced response to the pathogen treatment. This weak response has been shown before to be beneficial for the animal. Since individual animal differences were also considerably high, further studies are needed to elucidate the additional effect of the environment and of epigenetic or microRNA influence.

P052

Influence of Nutrition Manipulation during an Early Growth Stage on Glucose Metabolisms of Longissimus Muscle in Wagyu (Japanese Black)

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Introduction - Japanese Black cattle, or "Wagyu", are known to accumulate high levels of intramuscular fat. This experiment was conducted to clarify how early nutrition affected glucose metabolism in Japanese Black steers fattened on roughage.

Materials and methods - Japanese Black steers were randomly allocated into two groups. The high-energy group (Imp: n=12) received intensified nursing (maximum intake of 1.8 kg per day) until 3 months of age and was then fed a high-concentrate diet from 4 to 10 months of age. The roughage group (Cont: n=11) received normal nursing (maximum intake of 0.6 kg per day) until 3 months of age and was then fed only roughage (orchard grass hay) *ad libitum* from 4 to 10 months of age. From 10 months of age, both groups were fed only roughage (orchard grass hay) *ad libitum* until 14 months of age. All animals were then put onto the same pasture and grazed until 20 months of age, fed only roughage (orchard grass hay) *ad libitum* from 21 to 31 months of age, and then slaughtered at 31 months of age. Fresh tissue samples were biopsied from the *longissimus* muscle (LM) at 3, 10, 14, 20 and 30 months of age. Three genes relating to glucose metabolism, GLUT1 (glucose transporter 1), GLUT4 (glucose transporter 4) and G6PD (glucose-6-phosphate dehydrogenase), were investigated in each LM sample by qPCR analysis (Applied Biosystems StepOneSystem, Life Technologies Co., Ltd., USA).

Results and discussion - The gene expression changes with growth in the Cont group indicate basic glucose metabolism expression patterns during roughage feeding. G6PD showed different expression patterns with growth and levels between groups, which we suggest was disordered by high nutrition in the early growth stage. GLUT1 has the same expression pattern and levels for both groups and represents genetic regulation of the expression. GLUT4 has the same expression pattern with growth but different expression levels between groups. This is thought to be disordered by nutrition at particular stages. The changing patterns with growth suggest that the glucose metabolism pathway was affected by high levels of nutrition and its effects remain until slaughter. By further investigating the details of these changes in glucose metabolism, we hope to improve beef production from grass resources.

P053

The expression and localization of some lymphangiogenic VEGF family members in bovine corpus luteum

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The aim of this study was to evaluate expression and localization of the assumedly important lymphangiogenic factors VEGFC and VEGFD

and the receptor FLT4 in bovine corpora lutea (CL) during different physiological stages. In experiment 1, CL were collected in a slaughterhouse and staged (days 1-2, 3-4, 5-7, 8-12, 13-16, >18) of estrous cycle and month <3, 3-5, 6-7 and >8 of pregnancy. In experiment 2, prostaglandin F2α (PGF)-induced luteolysis was performed in 30 cows which were injected with PGF analogue on day 8-12 (mid luteal phase) and CL were collected before and 0.5, 2, 4, 12, 24, 48 and 64 h after PGF injection. The mRNA expression was characterized by RT-qPCR, the protein concentration by ELISA and localization by immunohistochemistry. All 3 factors were clearly expressed and showed significant changes during different groups and periods examined in both experiments. Protein concentrations of VEGFD and FLT4 were not detectable in early cyclic CL but increased to higher plateau levels during pregnancy. After PGF-induced luteolysis FLT4 protein showed an increase within 2-24 h after the injection. FLT4 localization in cytoplasm of luteal cells was relatively weak in early CL but increased in late CL, and especially in CL during pregnancy. During pregnancy a positive FLT4 staining in both the nucleus and cytoplasm of lymphatic endothelial cells in peripheral tissue was observed. In conclusion, our results lead to the assumption that lymphangiogenic factors are produced and regulated in CL and may be involved in mechanisms regulating CL function, especially during pregnancy.

New Application in qPCR

P054 – P071

P054

Novel mismatch suppression strategy enabling allele-specific cDNA synthesis and ultra-sensitive detection of RNA variants

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Although various DNA mutation detection assays can identify the presence of specific mutations, the expression level of the mutant alleles of coding genes may vary (Yan, et al., 2002) and affect the phenotype of cells or tissues harboring these mutations. As a result, a quantitative assay for analysis of mutant RNA transcripts could more accurately reflect the effect brought upon the cells or tissues by the presence of a specific mutation. In addition, such an assay to analyse mutations from RNA samples is also beneficial to either detection of clinically relevant mutations within viral RNA genomes, such as HIV drug-resistance mutations or detection of post-transcriptional changes of in RNA sequences (RNA editing).

We have utilized a moderately thermo-stable reverse transcription enzyme to develop a novel strategy, named ART-PCR (Allele-specific Reverse Transcription – PCR), for ultra-sensitive detection and quantification of mutant RNA in the presence of excessive amounts of the wildtype transcripts. Unlike conventional approaches, the ART-PCR assay achieves allele-specific priming in a hot-start cDNA synthesis utilizing a mutation-specific primer with a 5' tail of unrelated sequence and a competitive wildtype-specific primer without a tail (Fig. 1A). The mutation-specific cDNA products are selectively amplified and detected in a PCR reaction containing a common-locus forward primer and a discriminating reverse primer, specific to the tail sequence of the mutation-specific reverse transcription primer (Fig. 1B). The wildtype-specific cDNA product is not amplified due to the lack of a binding site for the reverse primer.

The ART-PCR assay was able to detect various mutant RNA transcripts of KRAS and BRAF genes in a 1000 - 10,000 fold excess of wild type transcripts and the quantitative detection was linear over a broad dynamic range. We demonstrate that the exceptional sensitivity of the ART-PCR assay is dependent on competitive primer extension during cDNA synthesis and is lost if the competitive wildtype-specific primer is either excluded from the reaction or replaced by a non-extendable oligonucleotide with the same sequence

Figure 1: Schematic of ART-PCR assay. The assay includes two steps: (**A**) Allele-specific cDNA synthesis competitively initiated by a mutation-specific primer with a 5' tail of unrelated sequence (dashed) and a wildtype-specific primer without a tail, and (**B**) selective PCR amplification/detection of mutation-specific cDNA product.

P055

New holistic Approach for easy and cost efficient Genotyping of small numbers of Genes.

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Despite the recent cost lowering of whole genome sequencing, it is still an unmet challenge to genotype a few nucleotides of interest in a fast, robust, inexpensive and scalable way. We will present a new holistic way of genotyping, using primers and probes based on modified DNA. The approach presented herein is closed tube assays that minimizes risk of cross contamination, runs on standard real-time PCR equipment, is scaled up fourteen times with use of inexpensive PCR instrumentation, can easily be multiplexed determining the three possible genotypes (homozygous WT, heterozygous or homozygous Mut) of up to at least four genes in each well, is very robust for input of sample material and is using standard procedures and reagents present in most laboratories.

We will introduce a novel, efficient, asymmetric PCR, which we call eAsymmetric™ PCR followed by an endpoint melt study using modified DNA probes called EasyBeacons™. The PCR reactions can be carried out on standard inexpensive PCR machines and read-out of the genotypes requires a ten minutes melt study post PCR on a real-time PCR instrument or temperature controlled fluorescence reader to evaluate the genotypes of up to four different genes per well.

eAsymmetric™ PCR is an alternative to LATE PCR that is easier to design and EasyBeacons are hybridization probes with increased affinity, sensitivity and signal-to-noise ratios.

P056

An RT-qPCR Automated Platform: Validation Methods for the Access™ Workstation, the Echo® Liquid Handler, and RealTime Ready™ Reagents

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Despite the growing value of RT-qPCR to quantify mRNA expression levels, large-scale application of the technique has been hindered by laborious multi-step processes with high reagent costs. Recent advances in reagent technology, such as the RealTime ready cell lysis reagents from Roche Applied Science, simplify the path from sample preparation to analysis. Utilizing these reagents in a miniaturized and automated workflow reduces overall operating costs and warrants the use of RT-qPCR on a larger scale. This study discusses the implementation and validation of a miniaturized and automated workflow with the Access workstation for high-throughput RT-qPCR. The validation of the individual steps and the overall workflow are presented.

P057

Using TaqMan Assay Followed By Melting Analysis Performed By New-Generation Intercalating Dyes

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Hydrolysis probes, also known as TaqMan (Applied Biosystems), are routinely used in qPCR diagnostics. The disadvantage of these probes is that they have to be designed for each target sequence separately, which makes the analysis more expensive. In order to reduce the costs, we can use the UPL probes (Universal ProbeLibrary; Roche), which contain locked nucleic acids and are of short length of just 8-9 nucleotides. The advantage of UPL probes is that they can bind to their targets with increased strength due to the incorporation of locked nucleic acids in their sequence. Their short length allows them to bind to several positions within the whole genome, which could be disadvantageous in some applications. Moreover, the point mutation can lead to the false negative or false positive results due to their short length. This problem can be overcome by using an intercalating dye which has a different fluorescent spectrum than UPL probe and binds unspecifically to any double stranded DNA Thus, we can get two fluorescent signals from one target sequence. Moreover, this intercalating dye can be used in either melting or HRM analysis. We developed the dual labelled two-step RT-qPCR for the detection of selected viruses. The UPL probe #138 was used for Louping ill and Powasan virus and UPL probe #15 was used for Chikungunya virus. The SYTO 61 Red Fluorescent Nucleic Acid Stain (Invitrogen) was added to all reactions as a second fluorescent reporter. The PCR profiles were used according to the UPL probe's manufacturer instructions. In all pathogens studied (Louping ill virus, Powasan virus and Chikungunya), there were observed no reciprocal overlaps in fluorescent spectra and the melting profiles were clearly distinguished. Our experiments showed that the combination of the UPL probe and SYTO intercalating dye in one qPCR assay represents a highly sensitive, specific, less time-consuming and cheaper method.

P058

Attachment Of Embryonic Stem Cells-derivedCardiomyocytes In Cultispher-S Microcarriers By Using Spinner Flask

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Embryonic Stem (ES) cells have the ability to differentiate under in vitro conditions into cardiomyocytes. A transgenic α-myosin heavy chain (α-MHC+)ES cell line was generated, exhibiting puromycin resistance and expressing enhanced green fluorescent protein (EGFP) under control of the α-MHC+ promoter. A puromycin-resistant, EGFP-positive α-MHC+ cardiomyocyte population was isolated with over 92% purity.

The cultivation of these cardiomyocytes, in macroporous gelatine microsphere beads in a spinner flask bioreactor has been studied. The average number of cultivated cells per microsphere was optimised after we specified the most suitable agitation conditions and the optimal timeframes of cultivation. Our study shows that 80 % of microspheres were colonised by cardiomyocytes under optimal conditions. Scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM) were used to show that the population of the beads was not limited to the microcarrier surface, but some cells invaded the inner surfaces of the microspheres as well.

The present findings demonstrate the successful culture of α-MHC+ cardiomyocytes in macroporous biodegradable microcarriers while maintaining the typical morphological and electrophysiological properties of cardiomyocytes. Our perspective significantly improves survival of grafted cardiomyocytes and thus help to overcome current limitations of cell replacement approaches.

P059

High Throughput Profiling Of Antigen Specific T Cells: Repertoires In Vaccine Or Autoimmunity

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Measuring and characterising the adaptive immune response to infections, vaccine or autoimmune pathologies is important to monitor disease progression or intervention success. The immune response can be described phenotypically, by means of T cell receptor diversity and qualitatively, by profiling expression of T cell- or activation state specific genes. We developed methods to analyse in high throughput the T cell receptor repertoire and gene expression of single T cells. Tetanus vaccine- and GAD65 autoantigen-responsive cells were profiled from different time points, cell subsets and subjects. We find high diversity of TCRs for antigen response within and between individuals. Despite this, antigen-responsive T cells could be tracked across time and T cell subsets. Gene expression profiles well reflected classic CD4+ T cell subsets. Remarkably, gene expression profiles can vary substantially amongst T cells expressing identical TCRs. This approach provides a powerful measure of immune response diversity and phenotype at the single cell level.

P060

Cost-effective real-time analysis by mediator probe (RT-)PCR

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Probe-based real-time PCR is used in molecular diagnostics due to its superior specificity and clinical sensitivity. High synthesis costs for sequence-specific dual-labelled detection probes are still one reason why researchers are reluctant when larger numbers of probes need to be ordered. In order to reduce costs we suggested a novel real time PCR method, the mediator probe PCR [1, 2]. It replaces fluorescently labeled hydrolysis probes by sequence-specific label free mediator probes (MP). Cleavage of the MP during amplification results in release of a mediator which is detected by a universal fluorogenic reporter (UR) oligonucleotide. The key to cost savings is that the same UR can be used for all assays and therefore can be ordered in large scale. This way oligonucleotide synthesis costs can be reduced to less than 40 % compared to the synthesis costs in hydrolysis probe based assays. In this work, performance characteristics of mediator probe PCR (MP PCR) were compared to hydrolysis probe PCR (HP PCR).

Method: Nucleic acids were extracted from two viral DNA targets (human papilloma virus-18, human adenovirus B7) and one viral RNA target (influenza virus B). MPs and the UR were designed according to design rules provided in [1]. Probe sequences of the MPs were based on the corresponding HP sequences and comprise the following differences: (1) HP: fluorescence quencher at the 3'-terminus; MP: phosphate group at the 3' terminus to avoid extension by polymerase activity; (2) HP: fluorophore at the 5'-terminus; MP: mediator sequence at the 5' terminus. As the mediator and the corresponding mediator hybridization sites at the UR can be standardized the same fluorogenic UR could be used for all assays.

Results: Analysis of serial dilutions of the DNA- and RNA targets revealed good agreement between MP and HP PCR: HPV18 (MP PCR $r^2 =$

0.999/HP PCR r^2 = 0.975), HAdV B7 (0.992/0.983), FluB (0.992/0.991). Amplification of 10^2 to10^6 copies HPV 18 DNA per reaction gave CVs of 55.1% to 9.9% / 38.3% to 10.7% and accuracies in the range of +21.6% to -8.1% / +19.4% to -9.8%. Amplification of 10^2 to10^5 copies HAdV B7 DNA per reaction gave CVs of 29.4% to 3.4% / 51.3% to 5.4% and accuracies ranging from +30.6% to -18.3% / +49.3% to -18.1%. Amplification of 10^4 to 10^7 copies FluB RNA per reaction gave CVs of 46.9% to 4.0% / 42.4% (4 out of 5 detectable) to 4.2% and accuracies ranging from +5% to -5% / +11% to -5%.

Using the novel MP (RT-)PCR detection of 3 different targets with one universal detection oligonucleotide, the UR, showed same performance characteristics as HP (RT-)PCRs. MP PCR can reduce oligonucleotide synthesis costs and is recommended where a multitude of probes with low batch size is required. This can be in research laboratories, during assay development or in low resource settings. Our current research aims at (1) extending the design rules and (2) increasing the degree of multiplexing.

P061

Differential expression of heat shock protein genes after temperature treatment in *Drosophila melanogaster* and *Megaselia scalaris* at different stages of development

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Induction or up-regulation of heat shock protein gene (*Hsp*) expression is an important physiological mechanism of thermal tolerance in insects. However, comparisons of thermal tolerance of laboratory-bred flies derived from distinct geographic locations have produced puzzling results. One of the reasons is that *Hsp* genes usually are considered to be a homogenous group induced or up-regulated by temperature shock. We studied the expression of *Hsp* genes in response to different heat shock temperatures in larvae, pupae, and imagoes of two species of locally occurring flies: *Drosophila melanogaster* (family Drosophilidae) and *Megaselia scalaris* (family Phoridae). The level of mRNA of *Hsp* genes was measured by Real Time PCR. Our results showed that the expression of *Hsp* genes is developmental stage-dependent, e.g., in *D. melanogaster* a treatment of larvae at 39°C increased *Hsp22* gene expression 53-fold, while in pupae and imagoes the increase was by 5.7 and 2.5 times, respectively. Optimal temperatures for *Hsp* induction/up-regulation were also different. While the temperature of 39°C caused the highest gene expression for *Hsp22* and *Hsp26* in larvae as well as for *Hsp27* in pupae of *D. melanogaster*, an optimal temperature for other genes and/or developmental stages was 38°C. In *M. scalaris*, e.g., the expression of *Hsp22* was constant in larvae, increased 2.7 times at 39°C in pupae, and was undetectable in imagoes. Evidently, temperature/stage expression of *Hsp* genes exhibit different patterns in *D. melanogaster* and *M. scalaris*.These results suggest that involvement of individual *Hsp* genes can be different at different thermal regimes but also species-specific, and different at various developmental stages.

P062

Evaluation of the Effect of Magnetic Nanoparticles as Additives on qPCR

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Nowadays, magnetic nanoparticles (NPs) play an important role in different technological areas such as electronics, energy and biomedicine[1]. Magnetic iron oxide NPs have interesting advantages such as controllable sizes, and the possibility of being manipulated by an external magnetic gradient which make their use for bioanalytical applications attractive. The development of faster, more sensitive and cheaper analytical methods can benefit from the use of these nanomaterials, however their use for PCR based applications should be investigated in detail to evaluate the effect of such NPs on the reaction efficiency, and therefore on the reliability of the results.

On the other side, the use of nanomaterials to improve and/or better control both end-point and quantitative PCR (qPCR) has shown an increased interest by the scientific community. Different NPs, such as carbon nanotubes, quantum dots, and gold, have been combined with PCR with interesting results. In some cases, they have demonstrated to improve PCR specificity and efficiency. Different mechanisms have been suggested to be involved such as mimicking of the function of singled-stranded DNA binding protein (SSB), interaction or binding DNA template and/or DNA polymerase helping to increase the specific annealing of primers to template DNA, or high thermal conductivity in the PCR mixture allowing faster ramping. However, other groups have found negative effects, such as inhibition of PCR reaction, fluorescent quenching on SYBR green I based detection systems, or DNA duplex destabilization. Effect of NPs on PCR and qPCR reactions have shown to be highly dependent on size and composition of NPs[2], but also on PCR conditions, and particularly on concentration and DNA polymerase used[3]. Therefore a case by case evaluation of the effect of different NPs on PCR must be performed.

We had evaluated the effect of 20-nm magnetite (Fe_3O_4) NPs functionalized with oleic acid and stabilized in aqueous solution by tetramethylammonium hydroxide surfactant on a previously designed and optimized qPCR for the detection of sesame DNA. The effect on the efficiency of the qPCR reaction on both MGB probe and SYBR green I chemistry methods was tested. High-resolution melt analysis (HRM) was used to study whether these NPs can affect melt behavior of the DNA.

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P063

PCR never got so Cyxi - Lyophilised Mol Biol Reagents

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Polymerase Chain Reaction is the standard amplification method for high confidence nucleic acid detection. The state-of-the-art includes Fluorogenic methods for homogenous real-time PCR and melting point analysis. High precision may be achieved through batched reagents that have been commercially available as frozen ready-to-go master products for over 15 years. The move to point-of-use integrated instruments has been an important driving factor in the development of new lyophilised processes. Fluorogenics Ltd is a new company focused on the development high performance PCR lyophilisation reagents. The Cyxi™ products eliminate the cold supply chain, are stable at ambient temperatures for up to two years, and have been developed for high throughput automated applications in the research and diagnostic sectors. Here we will present the key technical challenges and the practical application of the Cyxi™ products.

P064

Evaluation of a new plasma stabilisation technology for circulating cell-free DNA

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A wealth of scientific evidence has shown that the circulating, cell-free nucleic acids (ccfNA), present in human blood plasma, have great potential in providing diagnostic markers. Over the last years, it also became clear that this evolving molecular diagnostic field requires new pre-analytical tools and procedures in order to ensure valid results. When blood is drawn according to the standard methods, blood cells and nucleic acids are not stabilized which leads to two processes occurring over time (hours to days): (1) Nucleic acids may degrade due to the presence of nucleases in whole blood or the plasma fraction, respectively. (2) Blood cells (e.g. leukocytes) will die and disintegrate over time, releasing comparatively large amounts of genomic, chromosomal DNA into the plasma fraction. This will reduce the fractional concentration of the ccfDNA originally present in the samples and, most importantly, will dilute potential diagnostic targets such as a fetal DNA markers or rare tumor-derived DNA fragments. The presented study aims to demonstrate a proof of concept for a new stabilization technology which is under development within the SPIDIA project, both in healthy population and in clinical blood samples derived from pregnant women and oncology patients.

P065

Single blastomere expression profiling revealed distribution of maternal transcripts in oocyte and daughter cells during early embryogenesis of *Xenopus laevis*.

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Expression profiling of single cell is the most powerful tool to study cellular differentiation and specialization during early development. Understanding of the processes that lead to the formation of the embryonic body plan and to identification and description of the molecules that are included in the body plan formation, is essential for developmental biology. The oocyte of *Xenopus leavis* is first divided into an animal and a vegetal hemisphere and maternal mRNAs are asymmetrically distributed along this axis. The asymmetry is transferred to the daughter cells during cell divisions. The dorso-ventral axis formation and the left-right specification also occurs in the early steps of embryogenesis. Presented study is focused on expression profiles of chosen developmental genes in individual blastomeres from 8 to 32-cell stages of *Xenopus laevis*. Our objective was to find asymmetry that could be related to the dorso-ventral specification and/or the left-right axis formation.

P066

VideoScan - A Microscope Imaging Technology Platform for the Multiplex Real-Time PCR

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The quantitative real-time PCR (qPCR) is the central technology for the quantification of nucleic acids. The ability to perform multiplex quantitative real-time PCRs (mqPCR) in one vessel depends on parallel read-out and high sample multiplexing. Conventional mqPCRs are challenging due to the lack of multiple spectral non-overlapping reporter dyes and technical limitations of the detection systems. Recently we published a highly versatile microscopy platform, designated VideoScan, for the rapid and simultaneous analysis of various assay formats based on fluorescence microscopic detection. It uses standard commercial hardware components together with a modular in-house developed software package. Multiplex assays can be performed in solution or on solid-phases as microbead-based assays and cell assays using conventional consumables. The system has been proven to be applicable for multiplex qPCRs, nucleic acid hybridization assays and melting curve analysis [1]. As we proposed earlier [2] our microbead technology in combination with qPCR generates a powerful technology for multiplex quantitative PCRs. In the present work we demonstrate our VideoScan technology for microbead-based multiplex quantitative real-time PCRs. The assay employs the interaction between gene-specific capture probes bound to thermostable microbeads and mobile gene-specific degradable detection probes which report the amplification. Our system requires only two encoding dyes for encoding of multiple microbead populations and one reporter dye for microbead bound capture probes for the discrimination of at least twelve amplification reactions. We used the method for the quantitative detection of several human and bacterial genes. The assay is amplicon size independent, requires no alteration of the PCR product, employs a simple probe structure and has no background fluorescence. Early results indicate that the amplification efficiency is similar to reactions in solution.

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P067

Engineered DNA Polymerases for Future Applications

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DNA polymerases are used in a plethora of biotechnical applications, especially in the polymerase chain reaction (PCR), genetic cloning procedures, genome sequencing and in diagnostic methods. Highly processive and accurate DNA polymerases are desired for cloning procedures resulting in shorter extension times as well as more robust and high yield amplification. A higher DNA polymerase fidelity may increase the reliability of genome sequencing and diagnostic systems. Amplification of ancient DNA samples requires DNA polymerases with an increased substrate spectrum to efficiently overcome typical DNA lesions. To enhance efficiency of forensic DNA testing, DNA polymerases resistant to inhibitors from blood and soil allow PCR without prior DNA purification. Further improvements of DNA polymerases are required e.g. to meet the needs for real-time DNA single-molecule sequencing that relies on the ability of DNA polymerases to efficiently

process modified nucleotides. Overall, custom-made and artificially engineered DNA polymerases which are leading to more robust and specific reaction systems are of urgent need.

In the past we have developed DNA polymerase screening assays (*Angew. Chem. Int. Ed.* **2009**, *48*, 4625; *Angew. Chem. Int. Ed.* **2002**, *41*,3620;*Curr. Prot. Chem. Biol.* **2010**, 89) and developed DNA polymerases with interesting properties such as increased lesion bypass property (*Angew. Chem. Int. Ed.* **2007**, *46*, 3115), selectivity (*Chem. Biol.* **2007**, *14*, 185; *Angew. Chem. Int. Ed.* **2005**, *44*, 4712), discrimination between C and the epigenetic marker 5mC (*patent application*) as well as the propensity to incorporate artificial nucleotides (*ChemBioChem* **2010**, *11*, 1963). Here we report on our resent development of a highly thermostable DNA polymerase with reverse transcriptase activity that was developed on the basis of earlier discoveries (*Angew. Chem. Int. Ed.* **2006**, *45*, 7633; *Biotechnol. J.* **2010**, *5*, 224). We demonstrate the versatility of this MultiTask DNA polymerase in many applications including 'zero-step' reverse transcription qPCR, pathogen detection, and sensitive amplification from highly-damaged DNA.

P068

Optimization of experiments using bisulfite pyrosequencing for gene-specific DNA methylation analyses

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Bisulfite pyrosequencing is the gold standard for quantitative determination of local DNA methylation. Studies using this technique are often targeting the identification of differences in methylation states between different treatment groups. However, the possibility to detect a significant difference is limited due to natural biological variation between subjects and sample processing. Thus, in case the number of subjects is limited, it is important to investigate at which processing step replicates are most efficient to increase the precision. In order to address this issue, hierarchically arranged bisulfite pyrosequencing analyses were conducted with triplicates of animals, triplicates of tissue samples from which DNA was extracted (sampling), triplicates of bisulfite conversion reactions, triplicates of amplification reactions (high resolution melting qPCR) and triplicates of pyrosequencing analyses. Ten CpG sites of the homeobox A10 (HOXA10) gene were examined in porcine endometrial tissues. For data analysis, a nested analysis of variance (ANOVA) was performed to determine standard deviations (SD) and variance contributions of the intersubject variation and especially of all subsequent processing steps. The estimated total noise of the experiment amounted to a maximum SD of 2.1 % methylation (mean 1.0 % methylation). Among all experimental steps, the sampling procedure accounted for the largest part of the variance with a mean SD of 0.8 % methylation, explaining on average 61 % of the total variance. Both the amplification and the pyrosequencing step mainly contributed to the remaining variance, while bisulfite conversion explained only 2 % of the total variance. Thus, in order to increase measurement precision and to resolve most notably small methylation differences between experimental groups, it is recommended to perform sample replicates. In addition, an overall SD of 2 % methylation shows good sample, assay and handling quality, as well as reliability and accuracy of the bisulfite pyrosequencing method.

P069

In Silico Design And *In Vitro* Validation Of 6-Plex RT-qPCR Assays

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OBJECTIVES - Quantitative real time PCR (qPCR) is a gold standard technique for quantification of nucleic acids including mRNAs[1]. In this study our objective was to develop a bioinformatics program able to design TaqMan assays suitable for Multiplex Quantitative Reverse-Transcription PCR (MQ-RTPCR) and to validate them *in vitro* in 6-plex RT-qPCR assays.

METHODOLOGY - We developed a program that queries both Refseq NCBI[2] and Ensembl[3] databanks for transcripts sequence downloading. mRNA sequences from both databanks were used to build a multiple sequence alignment (MSA) using ClustalW v2[4] (default parameters). Using this MSA, we automatically identified conserved regions on which the Primers and Probes (P&P) should be designed upon. To do so, an assay design program called "SLv8" was built (SL=SantaLucia Salt correction formula for Tm computation[5]). It encapsulates Primer3[6] which allows both oligonucleotides design as well as their scoring. Using 21 assay design rules, SLv8 program was able to design a qPCR assay and a covering couple of primers producing a PCR amplicon used for the standard curve simultaneously. For each relevant gene published before[7], different candidates P&P were tested *in silico* (regarding their specificity and their thermodynamics compatibility) and *in vitro* on biological samples in 1-plex and 6-plex experiments.

RESULTS - For the vast majority of the genes, SLv8 was able to design assays meeting all set design criteria. In a second step, designed assays were checked manually for alternative transcripts coverage, and SNP presence. Assays performance was validated *in vitro* by preparing series of 5-fold dilutions. Assays tested in 6-plex gave PCR efficiency between 93% and 100% with R^2>0.998. The maximum difference between samples run in 1-plex and 6-plex was 0.45 Cq.

CONCLUSION - We have developed a MQ-RTPCR Assay Design Pipeline called "SLv8" which allowed successful design of singleplex assays that can be multiplexed. Several future improvements of SLv8 are already listed such as avoiding design on SNPs positions (based on automated analysis of polymorphisms data from dbSNP database[8]) and launching a sensitivity and specificity check of P&P using thermodynamics simulation of qPCR reaction.

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P070

Next Generation Enzyme: A Multifunctional DNA Polymerase for Ultrafast Reverse Transcription

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Gründungsinitiative Prolago Biotec, Universität Konstanz

Wild-type DNA polymerases have amazing features and render them extremely useful for biotechnology and diagnostic purposes. Nevertheless, we use directed artificial evolution to tailor them for perfection. We have achieved a major innovative step forward by advancing wild-type DNA polymerase Klentaq through directed evolution, presenting here a new generation of a multifunctional DNA polymerase. In addition to its natural DNA polymerase activity it comprises high reverse transcriptase activity and is thus able to reverse transcribe RNA and amplify DNA efficiently. As a result, we do not need an explicit reverse transcription step anymore, which enables a novel 'zero-step' reverse transcription (RT) PCR. Given the absence of an RT step, the enzyme allows an ultra-fast PCR assay, with an overall PCR time of less than 20 minutes. We demonstrated this with three human mRNA transcripts (beta actin *ACTB*, hypoxanthine-guanine phosphoribosyltransferase *HPRT1* and glycerinaldehyde-3-phosphate dehydrogenase *GAPDH*). Our new enzyme also allows transcription

analysis with a direct-from-cell PCR, and furthermore we successfully tested our enzyme for pathogen detection such as Influenza A virus. In comparison with commercially available one-step RT-PCR products, we do not use an enzyme blend or special additives such as manganese-ions. By using the powerful tool of artificial evolution and directed selection, we are also working on a DNA polymerase with strongly enhanced discrimination abilities, which is highly valuable for SNP detections and methylation specific PCRs. Additionally, we are developing a fast, reliable and bisulfite-free method for CpG methylation detection.

Prolago Biotec is a start-up initiative from theUniversityofKonstanz, originating from the research group of Prof. Dr. Andreas Marx. We develop innovative tools for research, molecular diagnostics and personalized medicine.

P071

Next Generation Enzymes for qPCR Applications

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Kapa Biosystems is a life science tools company focused on protein engineering using high-throughput molecular evolution technology.

After almost 25 years, the PCR market is still primarily serviced by only a few generalist enzymes isolated from thermophilic organisms. With little modification to date, these enzymes are inadequate for the multitude of increasingly specialist and demanding emerging PCR applications, including those recently highlighted since the emergence of Next Generation Sequencing (NGS) technologies.

We have established an *in vitro* high-throughput molecular evolution technology platform for the engineering of novel DNA-modifying enzymes for the life sciences, diagnostics and applied PCR markets. Based on two enabling technologies, emulsion PCR coupled with high-throughput functional screening assays, the platform allows us to overcome the major bottleneck of the molecular evolution approach to protein engineering, namely the ability to link the generation of large libraries of protein variants with sufficient screening power.

We have released four novel, engineered enzymes since September 2007.   Some of these have been incorporated into real time PCR products. The improved characteristics and applications of some of these real time PCR products will be discussed.

Digital PCR

P072 – P075

P072

Detection of Rare Somatic Mutations Using a Simplified, Specific Digital PCR Workflow with Zero Dead Volume.

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The detection and enumeration of low frequency somatic mutations is crucial to advancing the understanding of cancer. In tumor and other cancer samples, the wild-type loci typically makes up the large majority of the nucleic acid present. In conventional qPCR and sequencing, signal from this wild-type template obscures signal from the mutant loci, limiting sensitivity. Digital PCR reduces this signal-to-noise problem by partitioning the nucleic acid template to a point where each reaction vessel contains a low number of copies, or no copy at all. This vastly reduces the concentration mismatch between mutant and wild-type loci at the level of the individual reactions. Using a large number of reaction vessels with the assumption of Poisson distribution of sample, the average number of copies per reactions can be calculated for both mutant and wild-type templates. This results in a highly precise, specific, and sensitive method of detecting and quantifying DNA. We have developed the QuantStudio™ 3D Digital PCR System to enable a simplified digittal-PCR workflow. The workflow is amenable to utilizing 100% of the sample, maximizing achievable sensitivity for small samples, and the reactions are permanently sealed during loading, minimizing risks of cross contamination. We demonstrate the utility of this method using well characterized cell lines containing KRAS mutations known to be causative in multiple cancer types. DNA from these lines was titrated with wild-type DNA to give mutant concentrations as low as 0.025%.

P073

A Simulation Study To Assess The Variance Components In Absolute Quantification Using Digital Droplet PCR

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Droplet digital polymerase chain reaction (ddPCR) is a novel technology for the quantification of nucleic acids in samples. It exploits emulsion PCR to partition the sample's target DNA across approximately 20 000 droplets. Per droplet, PCR reactions are then thermally cycled to endpoint where its fluorescence level is measured. This fluorescence is transformed into a binary indicator of target DNA presence using a chosen cut-off for detection. The downstream data analysis assumes copy numbers in the droplets follow independent and identically distributed Poisson distributions with λ as expected value. The Poisson assumption allows to simply estimate the original number of copies in the reaction mix as the the estimated λ times the number of droplets. Three different standard dPCR data analysis approaches tackle this computation starting from replicates: a) by pooling replicates to derive a single estimate, b) as the arithmetic average of the estimates per replicate or c) as their weighted average following the volume in each replicate. The asymptotic (sampling) variance of the estimates can then be calculated for each approach, while for b) and c) we can additionally derive the between replicates variance component. In practice, the results will be obscured by technical and biological variability which leads, among other things, to different λ's between replicates. We constructed a simulation study for assessing the impact of different sources of variability on the accuracy and precision of the estimates. Plausible ranges for each source of variability are derived from real data and published results. We found that the copy number estimators performed reasonably well under mild departures from the assumptions and consistent results could be obtained in most cases. However, even small deviations from these assumptions lead to a clear underestimation of uncertainty when using asymptotic variance approximations. Adding between replicates variation, which is well below the maximum permissible pipetteering error, we found the standard deviation to be underestimated by 10 to 30% for samples with a reasonable number of copies, while coverage of the 95% CI dropped to some 83.5%. When added variability increased further, these numbers quickly inflated to underestimate the standard deviation by more than 80% (5 times too small) leaving a CI coverage as low as 25.4%. Since single samples as well as pooled replicates no longer contain retrievable information on the between replicates variance, we advice against pooling and stress the importance of replicates to obtain a good estimate of the initial copy number and its corresponding standard error.

P074

Digital PCR Modeling for Maximal Sensitivity, Dynamic Range and Measurement Precision

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The great promise of digital PCR is the potential for unparalleled precision enabling accurate measurements for genetic quantification. When maximal precision is desired, a challenge with an unknown sample is to perform the experiment at a dilution that supports the detection of one or multiple targets of interest at the required measurement precision. A mathematical framework can be used for modeling a digital PCR system with factors impacting precision such as the number of available reaction chambers, sample volume reduction (due to a variety of causes), and false negative/false positive rates. This framework is used to develop graphics showing the relationship between precision and the supported dynamic range. The impact of total input sample volume on the lowest limit of detection or sensitivity is also illustrated.

A set of graphics relating the effects of various system parameters serves as a powerful tool to estimate dilution factors and number of reaction chambers necessary to get to a digital answer with the desired precision. The model predicts an increase in supported dynamic range, at a given precision, for the same number of reaction chambers with the use of two dilution points (using half the number of reaction chambers for each dilution). This increase in dynamic range is obviously advantageous where continuous detection across an entire dynamic range is desirable (e.g., genetic quantification). The loss of half the number of reaction chambers to a second dilution point incurs a slight loss in the detectable concentration range at a given precision. However, this loss is more than offset by the gain in concentrations now detectable because of an overlapping effect of the second dilution point. Furthermore, beyond two dilutions, gains are not found to be as significant suggesting careful choice of fewer dilutions to optimally leverage available reaction chambers for a digital PCR system. The results also predict that it is possible to leverage the available reaction chambers to enable precise detection of two targets present at largely different proportions within a given sample by careful choice of dilution factors. A subset of the available chambers is dedicated to detecting the rare type and the remaining chambers are dedicated to detecting the wild type at a very different dilution.

The curves showing precision versus concentration will be used to plan two digital PCR experiments. The first one will demonstrate a gene expression quantification workflow across a given dynamic range using a set of samples spanning five orders of magnitude in expression levels. The second one will demonstrate how to target a particular detection precision for both the rare and wild type alleles, using a dual reporter SNP assay from Life Technologies.

P075

Next Generation Digital PCR Technology: A Simple, Chip-based Nanofluidic system for any benchtop.

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Digital PCR enables specific nucleic acid sequences to be accurately and precisely quantified without the need for a reference. This ability for absolute quantification is key to applications such as pathogen quantification and GMO detection. With the advent of novel nanofluidic PCR technologies, running the hundreds to thousands of reactions required for digital PCR has become feasible. To this end, Life Technologies has developed a new silicon chip-based digital PCR solution that can be rapidly loaded with little to no dead volume. At the heart of the nanofluidic system is a small chip that enables 20K reactions to be run on a single sample. Working with external collaborators, we will be demonstrating the performance and utility of the system across a wide variety of applications and sample types.

Non-coding RNAs

P076 – P081

P076

Differences of miRNA Expression and DNA Modification Profile in Undifferentiated and Differentiated Mesenchymal Stem Cells

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The ability to differentiate into various lineages (e.g. osteogenic, adipogenic) and undemanding culture conditions make pluripotent mesenchymal stem cells (MSCs) a potential tool for therapeutics and a convenient model for exploring cell pluripotency and differentiation mechanisms. MicroRNAs (miRNAs), a class of small noncoding RNAs, and epigenetic DNA modifications play an important role in cell differentiation and pluripotency. Thus, the present study aims at detailed characterization of miRNA expression profile and DNA modification marks, methylcytosine (5-mC) and hydroxymethylcytosine (5-hmC), in MSCs for identification of epigenetic marks specific to cell differentiation or maintenance of the pluripotent state.

The expression profiling of more than 1300 miRNAs in both undifferentiated cells and adipogenic or osteogenic lineages of MSCs was performed using hybridization-based microarrays (Agilent Technologies) and confirmed with TaqMan Low Density Arrays (TLDA, Applied Biosystems). Several miRNAs were identified to be downregulated (miR-31, miR-382, miR-410, miR-432) or upregulated (miR-30a) in differentiated as compared to undifferentiated MSCs. Furthermore, changes of miRNA expression were associated with the cell differentiation into specific lineage. For the quantitative analysis of 5-hmC in a set of pluripotency-differentiation genes (*SOX2*, *H19*, *NANOG, TERT*), restriction with MspI of glucosylated DNA was applied followed by qPCR. A noticeable increase in 5-hmC level was associated with osteogenic cell differentiation. Promoter methylation, analysed by means of methylation specific PCR (MSP), of genes *SOX2*, *H19*, and *NANOG* remained stable, while *TERT*became hemimethylated in differentiated MSCs.

Preliminary results of our study identified miRNA expression and DNA modification profiles characteristic to cell differentiation.

P077

A Comprehensive Analysis of mRNA and microRNA Expression in Retinoic Acid treated 3T3-L1 Preadipocytes

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Backround - Adipogenesis, the differentiation of preadipocytes to fat cells is one of the most studied topics of cellular differentiation. In this study, the effect of retinoic acid (RA), a potent modulator of differentiation, was tested in 3T3-L1 mouse preadipocyte. RA induces on the one hand the differentiation of chondroblasts, osteoblasts and myoblasts. On the other hand it inhibits the differentiation of preadipocytes to

mature adipocytes. Aim of the study was to decipher new aspects of adipocyte differentiation relating to retinoic acid signaling. For this, mRNA expression was measured by using high throughput transcriptomics like microarray technology (Affymetrix) and RT-qPCR. For microRNA profiling the nCounter mouse microRNA expression assay (NanoString) was used. However, direct and indirect interactions of mRNAs and microRNAs cannot be illustrated by these methods. Hence, an integrative analysis of mRNA and microRNA expression was performed via microRNA.org, TargetScanMouse and GEPS pathway analysis software tool (Genomatix).

Methodology / Principal Findings - During multiple experiments total cellular RNA was isolated from differentiating 3T3-L1 preadipocytes, which were cultivated in presence and absence of 3 µM retinoic acid. The cells were harvested at the time point 0h, 2h, 4h, 7h, 10h, 24h, 48h, 96h and after 12 days for differentiation control. The analysis of gene expression during the first 96 hours after differentiation induction is of utmost interest, because we were looking for immediate early genes. For gene expression profiling RNA samples were analyzed (EMBL, Heidelberg) by using the GeneChip Mouse Gene 1.0 ST Array (Affymetrix). Moreover, the microRNA expression subjected to RA treated and untreated 3T3-L1 cells were investigated by using the detection system of NanoString. The gene expression data were validated by comparing the results with RT-qPCR results of 14 genes including PPARg, CEBPa, CEBPb, the key regulators of adipogenesis. After statistical data analysis, RA specific gene and microRNA expression clusters were generated. Afterwards, software based analysis of pathways (Genomatix), which are influenced by RA were carried out. In conclusion, an integrative analysis of microRNA and mRNA expressed Data was performed by microRNA.org, TargetScanMouse and GEPS.

Results /Conclusions - To generate a comprehensive picture of metabolism in preadipocytes, it is important to get a better understanding of mRNA target regulation by microRNAs. For this, we carried out an integrative analysis of mRNA and microRNA expression in 3T3-L1 cells. Retinoic acid inhibits both the *cebpa* and *pparg* signaling pathway. In addition, the calveolin-1 and *VEGF* pathways are also regulated by RA. However, microRNA-143, a putative adipogentic relevant microRNA, is not significantly regulated by RA. During RA treatment the expression of *smad3*, *gata6* and *cenpm* are up regulated. Therefore, these three genes might be novel targets of RA.

P078

miRNA High-Throughput Sequencing in Endometriosis

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Introduction: Studies of eutopic and ectopic endometria have suggested microRNAs (miRNAs) are involved in endometriosis development, but the full miRNome of eutopic and ectopic endometria is a field that needs to be explored. In this study we used high-throughput sequencing to characterize miRNA expression in endometriosis foci by comparing adjacent healthy tissue to ectopic endometrium of the same patient. Also, our study aimed to reveal in what extent the miRNA expression profile in endometriosis foci differs from eutopic endometrium.

Material and methods: Deep sequencing technology was used to obtain miRNome data of 5 peritoneal endometriotic foci, 5 adjacent healthy tissues and 2 eutopic endometrial samples of two women with moderate-severe endometriosis. To test for differential miRNA expression between endometriosis foci, healthy tissue and eutopic endometrium, the edgeR package for R was used. miRNAs with adjusted p-value (p <0.05) and false discovery rate (FDR) < 0.1 were considered statistically significant. Differentially expressed miRNAs were further validated using qRT- PCR.

Results: Endometriotic foci and adjacent healthy tissue had similar miRNA expression patterns that differed largely from eutopic endometrium. Two miRNAs (both p-values< 0.001, FDR 0.004) were significantly up-regulated in endometriotic foci compared to adjacent healthy tissues. Validation analysis confirmed differential expression of these miRNAs in endometriotic foci.

Conclusions: The comparision between endometriotic foci and healthy tissues revealed two miRNAs that could play an important role in the pathogenesis of endometriosis. miRNA expression profile of endometriosis foci and adjacent healthy tissue is more similar compared to endometrial miRNA profile.

P079

MicroRNA in biofluids – a case study on urine and the impact of optimized protocols.

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mircroRNA are small regulatory RNAs playing an important role in cellular and developmental process and their expression pattern reflects tissue and celltype of origin as well as physiological state. These 20-23 nt long RNA molecules show a surprisingly high stability in typical clinical sample material (e.g. FFPE blocks, plasma, serum, urine, CSF etc.) making them prime candidates for biomarkers.

Exiqon has developed an LNA™-based microRNA qPCR platform for detection of microRNAs with unparalleled sensitivity, specificity, and robustness. With this platform microRNAs can be detected efficiently irrespective of their GC content and even from biofluids where microRNA levels are extremely low. The platform uses a single universal RT reaction to conduct full miRNome profiling, thus allowing high-throughput profiling of microRNAs from important clinical sources without the need for pre-amplification.

Exiqon scientists have profiled thousands of biofluid samples, including blood serum/plasma, urine, and CSF. Based on this expertise a specific qPCR panel containing the most relevant microRNAs for toxicology studies was selected and combined with microRNAs found in various biofluids, tissue-specific microRNA markers and reference gene candidates for circulating microRNAs.

Here we report a toxicology study demonstrating the use of microRNA as biomarkers in urine to monitor the effect of a potent nephrotoxin. Urine is a particularly difficult sample to work with due to low amounts of nucleic acids and high amounts of PCR inhibitors. We show how these additional challenges of low microRNA amount in samples like serum/plasma, urine and CSF can be met with optimized workflows from sample preparation to PCR.

P080

Identification of Differentially Expressed Genes and microRNAs in Myxoid Liposarcoma in order to Reveal New Therapeutic Target Structures

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Aims - Liposarcomas belong to the most common soft tissue tumours (10-15%), but therapeutic options are limited and specific agents with defined molecular target structures are not available. As myxoid liposarcoma is caused by a gene translocation leading to the formation of an aberrant transcription factor, expression of many genes may be altered in these tumours. Therefore, we implemented gene and microRNA expression analyses in myxoid liposarcoma in order to reveal key molecules and signal transduction pathways involved in tumour formation and progression. We aimed at identifying candidate genes and microRNAs that may serve as diagnostic or prognostic biomarkers or even as therapeutic target structures.

Methods - Whole genome and microRNA microarray analyses were performed on a well characterised cohort of tumour samples and fat samples as control. The results could be verified by qPCR. Expression of candidate genes was analysed by qPCR and immunohistochemistry in primary tumour samples. Furthermore, inhibition experiments in the myxoid liposarcoma cell lines MLS402 and MLS1765 were performed in order to examine the functional relevance of selected candidates.

In addition to their separate analysis, whole genome and microRNA microarrays were evaluated together using the bioinformatics package BIRTA (Bayesian Inference of Regulation of Transcriptional Activity). This analysis integrates mRNA and microRNA expression data to infer the activities of transcription factors and microRNAs.

Results - Among the significant differentially expressed genes and microRNAs we could identify interesting candidates with high clinical relevance such as the microRNAs miR-29 and miR-181 or the genes *TOP2A* and *FGFR2*. Together with other members of the FGF/FGFR family *FGFR2* was detected to be overexpressed in myxoid liposarcomas compared to fat tissue. Overexpression was confirmed by qPCR in the whole tumour cohort. FGFR2 expression in primary myxoid liposarcomas was also shown on protein level by immunohistochemical staining.

By BIRTA analysis transcription factors and microRNAs with differential activity in myxoid liposarcoma samples compared to fat tissue could be identified.

Conclusions - Our study revealed several new candidate genes and microRNAs in myxoid liposarcoma which represent starting points for the establishment of specific therapeutic strategies. As one approach we could demonstrate a potential role of FGFR signalling in the pathogenesis of myxoid liposarcoma.

P081

No Substantial Differences in Circulating microRNA Profile Throughout the Menstrual Cycle

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Circulating microRNAs (miRNAs) are promising biomarkers for non-invasive diagnosis of infertility and gynaecological diseases. In addition to different pathological states, several normal physiological variables can contribute to alterations in circulating miRNA expression levels. This study aimed to determine the plasma miRNA profile of healthy women during the menstrual cycle and to assess which circulating miRNAs are derived from blood cells. The plasma miRNA expression profiles in six healthy women were determined by quantitative real time PCR from four time-points of the menstrual cycle. miRNA deep sequencing was performed on blood samples of one healthy woman. Our results indicated that circulating miRNA expression levels in healthy women were not significantly altered by the processes occurring during the menstrual cycle. No significant differences in plasma miRNA expression levels were observed between the menstrual cycle time-points, but the number of detected miRNAs showed considerable variation among the studied individuals. miRNA sequencing revealed that majority of miRNAs in plasma are derived from blood cells. The most abundant miRNAs in plasma and blood were hsa-miR-451a and hsa-miR-486-5p, but a number of miRNAs were unique to both sample types. In conclusion, our data suggest that the changes in the female body during the menstrual cycle do not affect the expression of circulating miRNAs at measurable levels.

qPCR Data Analysis

P082 – P090

P082

How to do successful gene expression analysis using real-time PCR

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Reverse transcription quantitative PCR (RT-qPCR) is considered today as the gold standard for accurate, sensitive and fast measurement of gene expression. Unfortunately, what many users fail to appreciate is that numerous critical issues in the workflow need to be addressed before biologically meaningful and trustworthy conclusions can be drawn.

Here, we review the entire workflow from the planning and preparation phase, over the actual real-time PCR cycling experiments to data-analysis and reporting steps. This process can be captured with the appropriate acronym PCR: plan/prepare, cycle and report.

The key message is that quality assurance and quality control are essential throughout the entire RT-qPCR workflow; from living cells, over extraction of nucleic acids, storage, various enzymatic steps such as DNase treatment, reverse transcription and PCR amplification, to data-analysis and finally reporting.

P083

Simurealization Of Between Repeat Variability In Real Time PCR Reactions

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Many decisions today rely on real time quantitative PCR (qPCR) analysis, yet few attempts have hitherto been made to account for uncertainty on key outcomes due to various sources of variation in the amplification process. Besides influences of more obvious factors such as camera noise and pipetting variation, variability of efficiency within and between reactions may affect PCR outcomes to a degree not fully recognized by the experimenter nor decision maker. Based on the Full Process Kinetics method (FPK-PCR), we have developed a statistical framework that models the accumulation of amplification error, measurement error and other sources of variation as they contribute to the differences in fluorescence patterns (and derived parameters) within and between reactions.

First we created a database including repeated reactions (technical repeats) covering a wide range of template and inhibitor concentrations in

order to cover key components of variation in the data generation process. Starting from the FPK-qPCR statistical procedure that models evolution of efficiency in a single PCR reaction, we sequentially introduce key error components, to arrive at a model capable of reproducing variation patterns witnessed in the data while keeping parameter values within realistic bounds. This formed the basis of a simulation engine producing realistic qPCR reaction data patterns.

Most of the variation in Cq values was adequately captured by the statistical model in terms of error components involved in the intended qPCR process. However, to recreate the dispersion of the repeats' plateau levels while keeping the other aspects of the qPCR curves within realistic bounds, additional sources of reagent consumption (side reactions) enter into the model. The resulting simurealizations generate realistic potential qPCR data and form a powerful tool to evaluate and compare performance of qPCR analysis methods.

Lievens, A., Van Aelst, S., Van den Bulcke, M. & Goetghebeur, E. (2012) Enhanced analysis of real-time PCR data by using a variable efficiency model: FPK-PCR *Nucleic Acids Research*, 40, e10

Lievens, A., Van Aelst, S., Van den Bulcke, M. & Goetghebeur, E. (2012) Simulation of between Repeat Variability in Real Time PCR Reactions*PLoS ONE, Public Library of Science*, 7, e4711

P084

Advanced copy number variant analysis with qbase[PLUS]

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Copy number changes are known to be involved in numerous genetic disorders. Moreover, copy number polymorphisms of various sizes are thought to contribute to normal phenotypic variation and susceptibility to multifactorial disease. In this context, qPCR-based copy number screening may serve as the method of choice for targeted copy number screening as it has many advantages over alternative methods, such as its low consumable and instrumentation costs, fast turnaround and assay development time, high sensitivity and open format (independent of a single supplier). Here, we present several pilot experiments in which we performed targeted deletion screening in patients with human genetic disorders. Accurate copy number calling and objective interpretation was performed with an advanced module for copy number analysis integrated within qbase[PLUS]. The software allows the selection of more than one reference sample for accurate copy number calling. In addition, it provides flexibility with regard to the reference samples as these samples may have varying copy numbers. The identified copy numbers changes are visualized on a per sample basis and conditional bar colouring is applied for easy detection of deletions and amplifications. In summary, we provide guidelines for qPCR-based copy number screening and subsequent data-analysis to improve the quality and reliability of your results.

P085

The Neglected Role Of Statistical Weighting In Real-time qPCR Data.

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In a recent large-scale qPCR methodology comparative study (Ruijter et al., 2003) it was shown that although sigmoidal models describe amplification curves quite well, they can exhibit significant bias and low precision in a technical replicate regime. Direct extrapolation of N_0 from the sigmoidal model has been shown to be of no avail since the lower asymptote of the sigmoid essentially fits only noise. Hence, quantitation of initial template numbers must be based on realistic estimates of efficiency and *Cq* values, both of them commonly derived from defined location indexes of the curve such as the second derivative maximum.

With a few exceptions (Zhao et al., 2005; Goll et al., 2006) all to date published methodologies that employ linear or nonlinear fitting conduct these unweighted and thus assume homoscedasticity (homogeneous variance) for all replicate cycles. We will show that this assumption is fundamentally wrong (by analysing variances along the curves of a plethora of published datasets) and demonstrate some developed variance models that compensate for qPCR-typical variance structures. The application of these variance models as weights in the nonlinear fitting regime significantly improves the precission of efficiency and *Cq* estimates for sigmoidal models. Furthermore, it can be observed that errors in *Cq* values as commonly observed today largely depend on the number of parameters in the model and are significantly higher than one would assume from replication error alone. Interestingly, a significant proportion can be attributed to "error in x", i.e. the cycle-to-cycle error in efficiency during thermal cycling.

P086

Bioinformatics workflows with Snakemake

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Samples sizes, documentation purposes and reproducibility make workflow management indispensable in modern bioinformatics. Snakemake is a workflow framework that provides an easy to read text based definition language and a versatile execution engine.

Workflows are defined via rules that are applied to files. Rules can be generalized by wildcards, and may contain shell invocations (e.g. of bioninformatics tools) as well as Python and R code. Dependencies between rules are determined by the system, and the resulting graph of jobs can be automatically parallelized. The scheduling of job execution can be parameterized with respect to job priorities, used threads and size of input files. Together with a generic support for batch and cluster engines this turns Snakemake into an extremely scalable system where workflow definition is decoupled from the executing platform. We demonstrate the benefit of using Snakemake when modelling an NGS pipeline.

P087

Uncertainty Quantification In Real Time PCR Using Bootstrap

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In today's practice qPCR results are reported without measure of uncertainty even though several factors including copy number variation, camera noise and baseline effects influence the qPCR process and generate variability on key outcome measures. Ignoring this prohibits the recognition of reliable decisions based on qPCR results within well-understood error bounds. The most straightforward approach to estimating uncertainty on qPCR results requires running repeated reactions. Unfortunately, such technical repeats can be costly.

The Full Process Kinetics method (FPK-PCR) analyses all available qPCR data by modeling the qPCR process completely including up until

the plateau at the end of the reaction. As FPK-PCR exploits a wide range of the data, it lends itself to uncertainty assessment through a bootstrap approach. Bootstrap procedures resample residuals from the fitted model and recombine these values with the fitted values of the original model. When such `resampled data' reflect the level of variation that could occur in the qPCR reaction, uncertainty of the FPK-PCR estimates follows from their variation within bootstrap resamples. A key condition for valid resamples is interchangeability of residuals. As the FPK-PCR trades a simple model with interpretable parameters, for some lack of fit in certain areas of the full reaction, its residuals are not interchangeable. For the purpose of creating bootstrap resamples we thus use a nonparametric fitting method based on Fourrier filtering. This more complex fit yields the needed interchangeability of residuals and thus valid bootstrap resamples.

We evaluate the bootstrap procedure on a database containing extensive repeats of qPCR reactions with variation in initial copy number and in maximal reaction efficiency due to inhibition. We construct confidence intervals for the maximal efficiency of a PCR reaction using bootstrap. As an application we examine to what extent bootstrap uncertainty estimates better enable detection of inhibition.

Lievens, A., Van Aelst, S., Van den Bulcke, M. & Goetghebeur, E. (2012) Enhanced analysis of real-time PCR data by using a variable efficiency model: FPK-PCR *Nucleic Acids Research*, 40, e10

P088

Analysis of Data from Experimental qPCR Systems with RKWard

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Real-time quantitative PCR (qPCR) is a well established method for the precise quantification of nucleic acids. However, there is an ongoing development of novel device concepts for qPCRs, hybridization and melting curve analysis. This includes lab-on-chip systems or the recently published microbead-based VideoScan platform [1]. Besides dedicated hardware an easy to use software for data analysis is also a requirement during developmental processes. Experimental instruments usually collect data of different formats and quality. Analysis software must therefore be able to preprocess data of different origins. Preprocessing includes data import, removal of missing values, selection of data ranges, outlier removal, detection of background signal, data transformation and smoothing /normalization of data. Subsequently, the software must be able to analyze and quantify both, the amplification data and melting curve data. To automate this process we developed a set of plugins for the open source software **RKWard** [1]. **RKWard** is a GUI and an *Integrated Development Environment* (IDE) for statistical analysis with **R** which provides tools to manage and edit different types of data objects. Statistical procedures and plots were implemented using the easily extendable **RKWard**plugin architecture based on **ECMAScript**, XML, and **R** [2]. The plugins are enhanced by a set of functions for data preprocessing. Core features rely on the excellent *qpcR* package [4] for qPCR quantification and algorithms from the *MBmca* package [5] for melting curve analysis. **RKWard**provides all tools of **R** for an interactive data analysis and presentation, a dedicated output file and an output window for documenting the results and figures. The software aims to accelerate the development process and to enable easier collaboration between colleagues in the hardware and wetware disciplines.

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P089

Advantages of *Cy0* Quantification Using Spline Interpolation

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Background: Quantitative real-time PCR represents a highly sensitive and powerful technology for the quantification of DNA. Behind the current analysis of real-time PCR data, there is a largely unexplored area of experimental conditions that limits the application of the *Ct* method, the current gold standard. The *Cy0* method can be considered similar to *Ct*, but its main advantage is that it takes into account the kinetic parameters of the amplification curve and may compensate for small variations among the samples being compared. Despite the advantages of Cy0, in some cases Cy0 value is not correctly calculated; this can be due to: presence of drift in baseline region, non linear plateau, non horizontal plateau and ratio between flex ordinate and Fmax value major then 1/e (e=2.7182...). In the experimental runs showing these features, Cy0 quantification could be biased; this could be due to errors in non-linear regression procedure, as suggested by residual analysis.

Aim: The aim of the present study is to compare the *Cy0* method, based on the non-linear fitting of Richards' curve with the *Cy0* method obtained using cubic polynomial spline interpolation in quantitative in real-time PCR.

Material and Methods: Experimental session was performed using two different curve standard (HE and LE): The DNA standard consisted of a pGEM-T (Promega) plasmid containing a 104 bp fragment of the human mitochondrial gene NADH dehydrogenase 1 (MT-ND1) as insert. This DNA fragment was amplified for quantification by the high amplification efficiency (HE) primer pair (forward ND1H: 5'-ACGCCATAAAACTCTTCACCAAAG-3' and reverse ND2: 5'-TAGTAGAAGAGCGATGGTGAGAGCTA-3') and low amplification efficiency (LE) primer pair (forward ND1L: 5'-ACGCCATAAAACTCTTCACCAAAG-3' and reverse ND2: 5'-TAGTAGAAGAGCGATGGTGAGAGCTA-3'). Moreover, real-time PCR quantifications were carried out from skeletal muscle tissue using fine needle aspiration (FNA) coupled with Multiplexed Tandem real-time RT-PCR as reported in Guescini et al. [2007].

Results and Conclusions: In standard conditions we found that the Cy0 method (http://www.cy0method.org/), based on Richards curve fitting, was robust producing unbiased and precise quantification. Despite this, in presence of strongly asymmetric curve, or with drift in baseline or in plateau region, Cy0 obtained with spline interpolation showed a better quantification, reducing biases due to decline of goodness of fitting in non–linear regression. Hence, by using spline interpolation Richards fitting limitations can be overcome.

P090

ChIP-qPCR and qbase[PLUS] jointly identify a MYCN-activated miRNA cluster in cancer

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Chromatin immunoprecipitation quantitative PCR (ChIP-qPCR) is very well suited to assess and quantify direct binding of specific regulatory proteins to genomic DNA sequences. Unfortunately, data-normalization and accurate quantification appear to be a major challenge for many users.

Here, we demonstrate that ChIP-qPCR in combination with state-of-the-art real-time PCR data-analysis software enables convenient and reliable quantification. We applied ChIP-qPCR to assess binding of transcription factor MYCN to miRNA cluster 17-92, to a positive control target, MDM2, and to a negative control target region. ChIP-qPCR was performed in two MYCN-overexpressing neuroblastoma cell lines (IMR5 and WAC2) using SYBR Green I detection chemistry in a 384-well plate and signals were normalized based on the average abundance of three non-specific genomic regions in the ChIP samples using the qbase[PLUS] multiple reference gene normalization technology. Fold enrichment was calculated relative to the input sample (non-precipitated) and compared to that of a fourth non-specific region (negative control target). Using this approach we were able to demonstrate strong MYCN-binding to the positive control and the miR-17-92 cluster. In keeping with this, the expression level of the miR-17-92 cluster is substantially increased in primary neuroblastoma tumor samples, in which the MYCN gene is amplified and overexpressed.

The results confirm the power of ChIP-qPCR in combination with the data-analysis software qbase[PLUS] to study gene regulation.

Assay Optimisation, MIQE and Quality Control
P091 - P105

P091

Improving biological relevancy of transcriptional biomarkers experiments by applying the MIQE guidelines to pre-clinical and clinical trials

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The ''Minimum Information for the Publication of qPCR Experiments'' (MIQE) guidelines are very much targeted at basic research experiments and have to our knowledge not been applied to qPCR assays carried out in the context of clinical trials. We aim here at showing the use of the MIQE qPCR app (App store, Apple) to assess the MIQE compliance of one clinical and five pre-clinical trials. This resulted in the need to include 14 modifications that make the guidelines more relevant for the assessment of this special type of application. We also discuss the need for flexibility, since while some parameters increase experimental quality, they also require more reagents and more time, which is not always feasible in a clinical setting.

P092

Analytical and Clinical Validation of In House qPCR for Detection of Human Papillomavirus (HPV) using MIQE Guidelines and ISO 15189

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Introduction: The laboratories accreditated by ISO 15189 have some requirements regarding the validation of assays, specially the In House or developed by the laboratory. The MIQE guidelines has shed some light in this specially dark subject of validation of In House assays. By other hand, the recent revision of the medical device directives, states that health institutions are still exempt from appliyng the regulations for devices manufactured and used within the same institutions but they must comply with ISO 15189 or other equivalent recognised standard.

Aim: Validation of an In House qPCR assay for the detection of HPV

Methodology: The implementation of this In House assay was performed accordingly with the MIQE, namely the items "Sample", "Nucleic Acid Extraction", "qPCR target information", "qPCR Oligonucleotides", "qPCR protocol", "qPCR validation" and "Data analysis". Briefly, after an initial research in PUBMED, we selected a methodology described by Payan et al, using the primers SPF10 modiefied - all primers were modified with an extra 4 bases in order to allow hybridization at 60°C and a two-amplification step protocol at 60 and 95°C (qPCR). This set of primers were validated *in silico* and with anogenital samples; the nucleic acid extraction was performed with QIAMP MiniElute (QIAGEN). SiHa cells were used as a Positive control. The qPCR setup is executed in a robot (QIAgility) and the real time reaction was performed in AB 7900HT. All the data are analysed in a Excel Sheet developed by the laboratory.

Results: Using this methodology we were able to:

- Improve the methodology for the detection of HPV in cervical samples, regarding the previous methodology used (conventional PCR, primers GP5+/6+)
- Determine the analytical sensibility for the different HPV genotypes (HPV 16 and18: 5 IU/reaction)
- Determine the clinical sensibility (86%) and Negative Predictive Value (94%), using CIN2+ as cutoff for disease
- Implement an IQC program for the daily monitorization (MultiQC)
- Implement several EQA programs for peer comparion of our results (QCMD, NEQAS, WHO LABNET HPV)
- Submit this assay to accreditation by ISO 15189

Conclusions: The MIQE Guidelines are a set powerfull tools for design and implementation of In House qPCR assays.

References: Payan et al, J. Clin. Microbiol. March 2007, vol. 45 no. 3 897-901

P093

Removal of Contaminating DNA from Commercial qPCR Master Mixes

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QPCR is an immensely sensitive method for detection of DNA. For this reason it is often used in methods for detection and quantification of bacteria in both research and clinical diagnostics. The *Taq*-polymerases used in commercial master mixes are in general produced recombinantly in *E. coli* and are in most cases contaminated with bacterial DNA from the production. The presence of this DNA in qPCR master mixes reduces sensitivity and might cause false positives when the intention is to detect minute amounts of bacterial DNA.

The new qPCR Decontamination Kit from ArcticZymes offers a fast, easy and affordable solution for removal of contaminating DNA from commercial qPCR master mixes. It utilizes a double-strand specific DNAse (dsDNase) originating from Arctic Shrimp to digest contaminating DNA in qPCRs. Because of its double-strand specificity, the decontamination protocol can be conducted with primers and probe present. The dsDNase is completely and irreversibly inactivated at 60˚C in the presence of DTT, ensuring that any template added after inactivation is safe from digestion.

Here we show that the qPCR decontamination kit removes contaminating bacterial DNA from commercial master mixes to levels below detection limit of a 45 cycle qPCR, thereby ensuring flat NTC samples. We also show that the treatment does not impair the sensitivity of the qPCR. The protocol is completed in less than an hour, and is shown to be functional in both Probe and SYBR based mixes.

P094

A Quanatitative RT-PCR-based Approach To Assess cDNA Quality For Comparative Gene Expression Analysis

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BACKGROUND AND OBJECTIVE: Synthesis of high quality cDNA is an important pre-requisite for proper measurement of differential gene expression by qRT-PCR method. The RNA quality, reverse transcriptase (RT) enzyme efficiency, and priming strategy determine the homogeneity of cDNA synthesis across mRNA template. Although assessment of cDNA quality is highly desirable to obtain reliable gene expression data, it is not generally performed due to lack of suitable and widely applicable methods. We have developed a qRT-PCR based method that will allow researchers to assess cDNA quality in various mammalian species.

METHOD: Housekeeping genes are well suited for cDNA quality assay as their mRNA transcripts are relatively long and ubiquitously expressed. Two sets of PCR primers – one towards the 5' end and another towards the 3' end – were designed along with two sets of internal primers for several housekeeping genes in rat and dog. RNA samples isolated from two cell lines and various tissues from these species were assessed by Agilent Bioanalyzer, which provided RNA Integrity Number (RIN) values. cDNA samples were prepared from various RNA samples, using different reverse transcriptase enzymes (from various commercial manufacturers) and different priming strategies (oligo-dT, random hexamers, or a mixture of random hexamers and oligo-dT). The cDNA quality was assessed by measuring the normalized expression of the 3' and 5' amplicons of a selected housekeeping gene, *Pgk1,* by qRT-PCR using SYBR1 dye. A 3':5' expression ratio value close to 1 suggests a homogenous representation of 3' and 5' regions of mRNA and high cDNA quality.

RESULTS: Generally, greater distances between the locations of the 3' and 5'-end amplicons in a gene transcript resulted in higher 3':5' ratios. However, the 3':5' ratios of housekeeping genes in rat and dog samples were highly dependent on the cDNA synthesis protocol used. Synthesis of cDNA from the same RNA sample using reverse transcriptase from different suppliers resulted in products presenting widely varying 3':5' ratios. The oligo-dT+hexamer priming method resulted in 3':5' ratios closer to one, while random hexamer showed a 5'-end representation bias and Oligo-dT priming a 3'-end representation bias. Studies on heat-degraded RNA samples revealed that as the RNA quality (RIN value) decreased, the 3':5' ratio of cDNA increased.

CONCLUSIONS: We have developed a qRT-PCR protocol for cDNA quality assessment that can be easily adopted by experimenters. Based onour data, we suggest that cDNA samples presenting similar 3':5' ratio should be used for differential expression analysis. Applying cDNA quality assessment may further improve reproducibility and reliability of qRT-PCR data in toxicogenomic studies.

P095

Assessing the quality and quantity of genomic DNA using the Agilent 2200 Tapestation and genomic DNA Screentape

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The success of any genomic study depends primarily on the quality of the starting material, like genomic DNA (gDNA). The integrity of the extracted gDNA affects the downstream applications like microarray hybridization and next generation sequencing library construction. Since these are expensive and time consuming applications, a quality control (QC) of the genomic DNA has become highly recommended. The integrity of gDNA has traditionally been analyzed using agarose gel electrophoresis, which is manual, cumbersome and can involve exposure to hazardous chemicals like ethidium bromide.

The Agilent gDNA ScreenTape has been developed specifically for the electrophoretic separation, sizing and quantification of genomic DNA samples. This ready-to-use device, which runs on the Agilent 2200 TapeStation instrument, provides a reproducible QC method for analyzing the integrity and quantity of genomic DNA combined with the convenience of an automated system. With minimal sample preparation, automated loading and a variable throughput system, digital results can be presented as a gel image, data table and in an electropherogram view. The ability to overlap and compare electropherograms within the software enables the discrimination of sample quality across different degradation states, sample types and concentrations. We present data that shows the Genomic DNA ScreenTape can easily verify the integrity of gDNA starting material with high sensitivity, precision and accuracy.

P096

Development of a system based on SMART technology for robust transcriptome library preparation from small quantities of degraded sample

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By providing sequence data on millions of short DNA fragments in parallel, Next Generation Sequencing has revolutionized biomedical research. In particular, the technique has enabled RNA expression analysis over the entire transcriptome with superlative sensitivity and dynamic range. With this has come a drive to utilize smaller and smaller sample inputs—with the ultimate goal of analyzing the transcriptome of a single cell. Achieving this requires highly sensitive and robust sample preparation methods. One powerful method for cDNA preparation is

SMART™ technology (Switching Mechanism At the 5' end of the RNA Template), which utilizes the template switching activity of reverse transcriptase to enable the direct addition of a PCR adaptor to the 3' end of the first-strand cDNA; thus avoiding inefficient ligation steps. The result is a single-tube protocol that greatly enhances library amplification efficiency as compared to traditional technologies, while minimizing the chance for contamination or degradation of the sample, making it ideal for library preparation from small amounts of starting material. Indeed, the currently available SMART kit allows researchers to readily obtain high quality data from as little as 100 pg of total RNA. And recent publications indicate that this technology is effective in single cell analysis. Although the SMART system provides many benefits, one drawback of the current system is the inability to work with samples comprising degraded RNA, owing to the method's dependence on an oligo dT primer for first-strand synthesis. In many samples where input amount is low—e.g., FFPE specimens—the starting RNA is often degraded. Accordingly, we have adapted the SMART protocol to work with degraded starting material, even with starting amounts as low as 10 ng total RNA. Data will be presented outlining various optimization steps, including the use of random primers, as well as methods for both purifying RNA from FFPE sections and a new method of depleting rRNA. In addition, the use of novel polymerase formulations to further enhance yield in the amplification step has also been investigated. These newer formulations are compared to other currently available enzymes with respect to total DNA yield, mutation rate and sample bias. Based on these modifications, we have developed a system that is capable of generating cDNA libraries for transcriptome profiling from FFPE samples starting with as little as 10 ng of total RNA.

P097

Nucleic acid purification with magnetic particle processor prior to qPCR

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Nucleic acid purification is an essential step before many common downstream assays, including PCR and real time PCR. DNA and RNA purification can be easily automated using magnetic particle technology. Thermo Scientific KingFisher magnetic particle processors are fast and efficient purification systems offering consistent results and hands-free time instead of laborious manual processing. For a medium-throughput laboratory, Thermo Scientific KingFisher Duo offers flexible system with wide processing volume range. KingFisher Flex is high-throughput instrument enabling purification from up to 96 samples per run. Combining the KingFisher Flex with Thermo Scientific Multidrop Combi for reagent dispensing automates the process even further. For downstream analyses Thermo Scientific PikoReal Real Time PCR instrument offers a unique system with excellent thermal performance, high sensitivity camera and five detection channels.

DNA and RNA was purified from human blood samples, with the KingFisher Flex and KingFisher Duo together with KingFisher Kits. Thermo Scientific Multiskan GO was used for DNA and RNA detection by measuring the absorbances. Genomic DNA elutions were used for an allelic discrimination study and a gene expression study was performed from total RNA elutions with the PikoReal Real Time PCR System. The results were analysed with the PikoReal Software.

The KingFisher instruments in combination with the KingFisher kits constitute an exceptional purification system for obtaining excellent yield and purity of DNA and RNA with high-consistency of parallel samples. Easy to use PikoReal Real Time PCR System provides fast and sensitive qPCR analysis in an accessible format.

P098

Reference Gene Selection And Validation For HT29 And VK2/E6E7 Human Epithelial Cell Lines Treated With Probiotic And Pathogenic Bacteria

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Introduction: Currently, the real-time quantitative polymerase chain reaction (qPCR) is the most sensitive tool for evaluating relative changes to gene expression levels, however as a result of its sensitivity an appropriate method of normalisation must be used to account for any variation incurred in preparatory experimental procedures. This variation may result from differences in amount of starting material, quality of extracted RNA, or in the efficiency of the reverse transcriptase or polymerase enzymes. Selection of an endogenous control gene is the preferred method of normalisation, however this selection is often made without proper validation of the gene's appropriateness for the study in question. Although there has been an increase in reference gene validation studies, to our knowledge, little has been conducted for studies involving relationships between probiotic bateria and host cells. The ability of commensal bacteria to influence gene expression in host cells under the influence of pathogenic bacteria has already been demonstrated, and investigation of the extent of this interaction is important to understanding how bacteria can be used as probiotics in the future. In this study we determine suitable reference genes for determining ge ne expression changes in human mucosal cell lines after bacterial treatment.

Methods: HT29 (colonic) and VK2/E6E7 (vaginal) human mucosal epithelial cells were treated with *Lactobacillus acidophilus, Lactobacillus rhamnosus* and heat killed *Escherichia coli.* We analysed samples using qPCR and applied four different algorithms (genorm[PLUS], BestKeeper, Normfinder, and comparative ΔC_q) to evaluate nine different genes as to their suitability as endogenous controls.

Results: The best reference gene candidates were different for each cell line, despite undergoing the same treatments. This confirms the importance of cell and tissue specific reference gene validation. We found phosphoglycerate kinase 1 (*PGK1*) to be most appropriate for HT29 cells, and peptidylprolyl isomerase (*PPIA*) to be the best choice for VK2/E6E7 cells. In both cell lines reference stability would be improved by use of multiple endogenous controls.

Conclusions: This study provides recommendations for stable endogenous control genes for use in further studies involving HT29 and VK2/E6E7 cells after bacterial challenge.

P099

Optimization of the DNA analysis workflow by applying an automated CTAB buffer-based DNA extraction prior to qPCR

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The amount of genetically engineered (GE) plants grown worldwide has increased year by year since commercialization in 1996. GE crops imported to the EU have to be authorized in order to be placed on the market (zero tolerance policy). This leads to an enormous effort for food, feed and seed surveillance authorities.

Surveillance of food, feed and seed is commonly performed by DNA analysis. DNA extraction is often accomplished by a manual and time consuming CTAB buffer-based DNA extraction. It often contains harmful substances like chloroform or phenol. In order to optimize this DNA analysis workflow we developed a CTAB buffer-based automated DNA extraction protocol.

We altered several extraction and chemistry parameters like incubation time, incubation buffer, binding buffer, initial sample amount, elution

buffer, additional enzymes and additives. The optimal protocol was further compared to manual CTAB buffer-based and Wizard-based DNA extraction protocols.

For each extraction procedure, DNA from corn, soybean and rapeseed samples containing 0.033 % gm material was extracted twelve times. Yield and purity were compared by NanoDrop and PicoGreen analyses and quantitative real-time PCR was applied in order to check the applicability of the extraction protocol for the official seed surveillance.

Higher yields and high DNA purity were achieved using the Maxwell® 16 instrument. Cq values were lower or at least comparable using the automated DNA extraction for both the reference genes and the event-specific detection assays. Quantitative real-time PCR analyses revealed no PCR inhibition, which was checked by analysing several dilution series. The applicability was also shown by analyses of routine samples from the official seed surveillance.

The development of this automated CTAB buffer-based DNA extraction protocol led to the production of a new automated DNA extraction kit for food, feed and seed by Promega GmbH. Currently, we adopt this protocol for DNA isolation from honey samples.

P100

MIQE in colic microbiota monitoring for clinical and preclinical trials

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MIQE is becoming more and more popular because of its focus on biological relevancy. Therefore, several studies are trying to implement the MIQE guideline. As a part of this trend, we wanted to be amongst the first researchers using the MIQE guideline for a clinical trial involving molecular biology.

Since the MIQE guideline is rather a blue print than a norm, it may take advantage of suggestions. Taking into account the particular setting that is a clinical trial, we made some propositions of adjustments concerning the handling of many samples.

P101

Quality Control of DNA from Formalin-Fixed Paraffin-Embedded and Fresh-Frozen Tissues Prior to Target-Enrichment and Next Generation Sequencing

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There are over 400 million formalin-fixed paraffin-embedded (FFPE) tissue samples archived in biobanks worldwide. These diseased and normal tissue collections are valuable resources for molecular genetic studies. However, the challenges of DNA extraction from FFPE tissues, including formaldehyde cross-linking, degradation, and mixtures of single-stranded and double-stranded DNA, result in low yields of usable material for downstream assays. Hence, quality control of samples is critical prior to costly experiments, such as next generation sequencing. On-chip and automated electrophoretic devices were evaluated for the characterization of FFPE and fresh-frozen DNA samples prior to and during target-enrichment and next generation sequencing workflows.

P102

High Throughput Miniaturized PCR using the Echo® 525 Liquid Handler

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Quantitative PCR (qPCR) has become widely prevalent across many disciplines throughout drug discovery. Advances in qPCR detection technology to include 384- and 1536-well plate formats have enabled researchers to increase throughput while decreasing reagent costs. To ensure high data quality, the liquid handling employed in such low-volume reactions must be precise and accurate. Tipless, touchless acoustic droplet ejection with the Echo® liquid handler eliminates the cost of disposable tips and wash cycles and improves workflow by simplifying assay setup. This study utilized new transfer methodologies to greatly reduce transfer times. Precision for the resulting quantification curves across 384- and 1536-well plates was excellent with standard deviations less than 0.25 and CVs less than 2.0%. Tests with positive and negative controls dispensed into alternating wells revealed zero cross-contamination. The results confirm the advantages of using the Echo liquid handler for preparing high-throughput miniaturized qPCR in both 384- and 1536-well formats.

P103

Quality control of RNA using the Agilent 2200 TapeStation system

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RNA has become the focus of recent investigations in understanding their biological roles in developing novel therapeutic schemes including RNAi. High quality RNA is a critical for the success of downstream experiments. In the present work Agilent 2200 TapeStation is used to assess the quality of RNA samples. Twenty one different eukaryotic RNA samples extracted from various tissue sources with varying quality was analysed, including six serially heat degraded RNA. The samples were analysed using R6K ScreenTape (R6K) and High Sensitivity R6K ScreenTape (HS R6K), consisting of 16 individual gel lanes. The performance was benchmarked against industry standard 2100 Bioanalyzer. TapeStation indicates RNA quality by RIN^e (RIN equivalent) with value between 1 and 10, where 10 is the highest quality RNA. A positive correlation between both systems with R^2 of 0.9878 for RNA 6000 Nano and R6K and R^2 value of 0.9474 for RNA 6000 Pico and HS R6K was obtained. The RIN^e reported by TapeStation shows <4% deviation from RIN for R6K and <7% for HS R6K. TapeStation shows greater reproducibility (RIN^e) with CV <3% compared to <6% in Bioanalyzer. Automated system of high throughput capabilities up to 96 samples with constant cost per sample makes TapeStation an ideal system for RNA quality assessment.

P104

Quantification of GMO at a Level of 0.1 % – A Statistical Approach Using Frequency Distribution

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According to Regulation (EC) No 619/2011, trace amounts of not authorised genetically modified organisms (GMO) in feed are tolerated within the EU, if certain prerequisites are met. Amongst other things, an application for authorisation pending with the European Food Safety Authority (EFSA), a specific detection method validated by the EU Reference Laboratory for GM Food & Feed (EURL-GMFF), and the availability of certified reference material are required. Tolerable traces must also not exceed the so-called 'minimum required performance limit' (MRPL), which was defined to correspond to 0.1 % mass fraction. Currently, 13 GMO events fulfil these requirements (EU Register of authorised GMOs; 21.01.2013).

Because of this new regulation, trace amounts of not yet authorised GMO (and some GMOs whose approvals have expired) in feed have to be quantified following the qualitative detection of such GMOs in extracted genomic DNA from those samples. The results of quantitative analysis can imply severe legal and financial consequences for producers or distributors of feed, the quantification results need to be utterly reliable.

Generally, random and systematic errors influence all measurement results. This holds also true for quantification of GMOs at all levels —not only at 0.1 %. Quantification of GMO adds further obstacles to the measuring procedure: After quantitative real-time PCR of both transgene and a species-specific reference gene, the mass fraction has to be calculated considering the (assumed) zygosity of the plant tissue(s) and plant species under investigation.

In the context of a scientific project sponsored by the Bavarian State Ministry of the Environment and Public Health (StMUG), we developed a statistical approach in order to investigate the experimental measurement variability within one 96-well PCR plate. The approach visualises the frequency distribution as mass fraction resulting from different combination of transgene and reference gene Cq values. One application is the simulation of the consequences of varying parameters on measurement results. Parameters could be for example replicate numbers or baseline and threshold settings, measurement results could be for example median (class) and relative standard deviation (RSD_r). All calculations were done using the functions of MS Excel.

The aims of the on-going study are scientifically based suggestions for minimising of uncertainty of especially in —but not limited to— the field of GMO quantification at low concentration levels.

P105

Gene Expression Profiling: qPCR Toolkit for Quality Control

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Gene expression profiling is exceedingly important tool in functional genomics research and in drug discovery. The general trend in the field is towards higher throughput of less complex samples. Nowadays studies are appearing where expression of tens of genes is measured in hundreds of individual single cells, to be able to characterize cell subtypes and its roles. To identify subpopulations of astrocytes, to define them in molecular terms, and to determine the biological progress of astrocyte maturation on single cell level we developed and optimized protocol for high-throughput gene expression profiling using qPCR together with a system of quality control: ValidPrimeTM - control for genomic DNA background, Interplate calibrator - tool, which is used to compensate for inter-run variation in qPCR and Universal Spike (RNA/DNA), which can be used to detect presence of inhibition in samples of any living species or monitor losses and yield of isolation, or check the itegrity of mRNA during sample processing, storage and transporation.

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