qPCR & NGS 2015

7th international qPCR & NGS Symposium

Industrial Exhibition & Application Workshops

"Advanced Molecular Diagnostics for Biomarker Discovery"

Event Proceedings

Updated Online Version

ISBN 9783000488054

Scientific coordination: Michael W. Pfaffl
Physiology, Freising – Weihenstephan
TUM School of Life Sciences
Weihenstephaner Berg 3
Technical University Munich (TUM)
85354 Freising
Germany

www.qPCR-NGS-2015.net

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Welcome to Freising!

Dear symposium participants,

On behalf of the organisation committee and the scientific board of the conference it is a great pleasure to welcome you to the **7th International qPCR & NGS 2015 Event**, taking place 23rd to the 27th March 2015. The focus of the symposium is

Advanced Molecular Diagnostics for Biomarker Discovery

The symposium is based on **77 lectures and 76 posters** presented by international recognised experts in their application fields. The emphasis will be on unbiased, didactic and scientific information exchange. Leading academic researchers and industrial contributors in the field will participate in the symposium, which will be an arena for fruitful discussions between researchers of different backgrounds. The Symposium Talks, Poster Sessions, Industrial Exhibition and associated qPCR & NGS Application Workshops offer an overview of the present knowledge and future developments in qPCR, next generation sequencing and gene expression measurement technology and its wide applications in research. The scientific talk and poster contributions are published in the qPCR & NGS 2015 Proceedings 'in hand' (ISBN 9783000488054). Presented oral presentations will be recorded and made public in autumn 2015 via the **www.eConferences.de** streaming platform together with around 200 talks from earlier conferences from qPCR 2010 onwards.
Three **Poster Sessions** will be shown from Monday evening till Wednesday early afternoon in the poster exhibition hall below the foyer. The new open access journal **Biomolecular Detection and Quantification** (BDQ) is proud to sponsor three **'BDQ Young Scientist Poster Awards'** for the best posters presented.
Parallel to the scientific symposium an **Industrial Exhibition** takes place where around 40 international companies will be presenting their newest qPCR, dPCR and NGS services and technologies.
The symposium is followed by five **qPCR & NGS Application Workshops** taking place March 26th and 27th powered by TATAA Biocenter, Bio-Rad, Qiagen, Genomatix and other leaders in the field.

Scientific Sessions Topics:

- Advanced Molecular Diagnostics
- Next Generation Sequencing & NGS data analysis
- Digital PCR
- Non-coding RNAs
- Biomarker Discovery & Circulating Nucleic Acids
- Microgenomics & Single-Cell Diagnostics
- Optimisation, MIQE & QC strategies in qPCR
- qPCR BioStatistics & BioInformatics
- Molecular Diagnostics in Agriculture, Veterinary Medicine, Food & Environmental Science

The scientific organization is managed by international well-known scientists in the field of molecular diagnostics:

Stephen Bustin	Prof. of Molecular Medicine, Faculty of Health, Social Care & Education, Anglia Ruskin University, UK
Mikael Kubista	Prof. of Biotechnology, BTU, Czech Academy of Sciences & TATAA Biocenter, Sweden
Vladimir Benes	PhD, Head of the Genomics Core Facility at EMBL, Heidelberg, Germany
Jim Huggett	PhD, Science Leader, Nucleic Acid Metrology, LGC, London Twickenham, UK
Jo Vandesompele	Prof. at the Center of Medical Genetics, University of Ghent, Belgium
Michael W. Pfaffl	Prof. of Molecular Physiology, TUM School of Life Sciences, Weihenstephan, Germany

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

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

qPCR & NGS 2015 Event Agenda Overview

Online agenda HTML => http://agendaHTML.qPCR-NGS-2015.net Online agenda PDF => http://agendaPDF.qPCR-NGS-2015.net

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Arrival & Registration</td></tr>
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Arrival & Registration
&
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<tr><td>14:00 – 18:30
Biomarker Discovery &
Circulating Nucleic Acids</td><td>14:00 – 18:30
MicroGenomics &
Single-Cell Diagnostics</td><td>14:00 – 18:00
Industrial Exhibition
in Foyer</td></tr>
<tr><td colspan="4">19:30 – 24:00 Symposium Gala Dinner
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MIQE & QC in qPCR</td><td>8:30 – 12:30
qPCR BioStatistics
& BioInformatics</td><td>8:30 – 14:00
Industrial Exhibition
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Molecular Diagnostics
Agi, Vet, Food & Env</td><td rowspan="2">14:00 – 17:00
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Michael W. Pfaffl</td><td></td></tr>
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26th March

Friday
27th March</td><td colspan="3">Seminar rooms S1 - S3 & Computer seminar rooms PU26, PU34A & PU36
9:00 - 17:00
qPCR, dPCR & NGS Application Workshops:
• Basic real-time qPCR Application Workshop (2-days) hosted by TATAA Biocenter
• qPCR Data Analysis Workflow: from instrument data to interpretation (2-days) hosted by TATAA
• digital PCR (2-days) hosted by Bio-Rad
• NGS data analysis workshop (2-days) hosted by Genomatix
• “Sample to Insight” Analyzing and interpreting the biological meaning in NGS data (2-days) hosted by Qiagen</td></tr>
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Agenda -- qPCR & NGS 2015

Sunday 22nd March 2015

12:00 – 18:00 Built-up for Industrial Exhibition

15:00 – 18:00 Arrival & Registration
Poster Setup

Monday 23rd March 2015

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

Stephen A. Bustin
Justion O Grady
Jim Huggett
Editors "Biomolecular Detection & Quantification"

Advanced Molecular Diagnostics 1

Monday, 23/Mar/2015:
10:30am - 12:30pm
Session Chair:
Stephen Andrew Bustin
Michael W. Pfaffl

Location:
Lecture hall 14

Presentations

10:30 **Keynote Lecture:**

PCR in less than 30 seconds: Efficient, specific amplification with increased primer and polymerase.

Jared S Farrar[1], Carl T Wittwer[2]
[1]Virginia Commonwealth University; [2]University of Utah; carl.wittwer@path.utah.edu

11:00 **Laser-driven PCR for ultrafast DNA detection**

Lars Ullerich, Federico Bürsgens, Joachim Stehr
GNA Biosolutions GmbH, Germany; ullerich@gna-bio.com

11:30 **Keynote Lecture:**

Digital PLA (Proximity Ligation Assay)

Harvinder Singh Dhillon[1], Christina Greenwood[1], Gemma Johnson[1], Mark Shannon[2], Doug Roberts[3], Stephen Bustin[1]
[1]Anglia Ruskin University, Chelmsford, Essex, United Kingdom; [2]Thermo Fisher Scientific, Sunnyvale, CA, USA; [3]Formulatrix, Bedford, MA, USA;stephen.bustin@anglia.ac.uk

12:30 **New advances in sequencing-based technology in Clinical Application and Molecular Diagnosis**

Fang Chen, Ya Gao, Hui Jiang, Yanyan Zhang
BGI-Shenzhen, China, People's Republic of; fangchen@genomics.cn

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

Advanced Molecular Diagnostics 2

Monday, 23/Mar/2015:
2:00pm - 6:00pm
Session Chair:
Justin O'Grady
Mikael Kubista

Location:
Lecture hall 14

Presentations

14:00 **Pathogen DNA enrichment strategies for nanopore sequencing based infectious diseases diagnostics.**

Justin O'Grady
University of East Anglia, United Kingdom; justin.ogrady@uea.ac.uk

14:30 **Differentiation of Identical Twins using Next Generation Sequencing**

Burkhard Rolf
Eurofins, Germany; BurkhardRolf@eurofins.com

15:00 **Mobile applications for qPCR**

Jo-Ann Stanton
Ubiquitome, New Zealand;
jo.stanton@anatomy.otago.ac.nz

15:30 **"NanoString - The Next Step After NGS"**

Michael Rhodes
NanoString Technologies; mrhodes@nanostring.com

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

16:30 **Optimized Targeted NGS With Multiplex Competitive PCR Amplicon Libraries for Reliable Diagnostic Testing of Cancer FFPE Samples**

Jiyoun Yeo[2], Erin Crawford[2], Tom Morrison[1], Xiaolu Zhang[2], Luke Stewart[3], James Willey[1,2]
[1]Accugenomics, Inc., United States of America; [2]University of Toledo College of Medicine; [3]Fluidigm, Inc.; james.willey2@utoledo.edu

17:00 **Whole Exome Sequencing for Detection of Genetic Variants to Predict Chronic Lung Allograft Dysfunction**

Markus Schmitt
GATC Biotech AG, Germany; m.schmitt@gatc-biotech.com

17:30 **Single Cell Analysis of Nuclear Receptor Transcription**

Michael Mancini
Baylor College of Medicine, United States of America; mancini@bcm.edu

18:00 – 22:00 **Evening Poster Session**

18:00 – 22:00 **Networking Reception in Industrial Exhibition**

Next Generation Sequencing 1

Monday, 23/Mar/2015:
2:00pm - 6:00pm
Session Chair:
Jan Hellemans
Steve Lefever

Location:
Lecture hall 15

Presentations

14:00 **Keynote Lecture:**

Next-Generation RNA-Seq

Gary Schroth
Illumina, United States of America; gschroth@illumina.com

14:30 **Targeted resequencing and variant validation using pxlence PCR assays**

Steve Lefever[1,2], Frauke Coppieters[1,2], Daisy Flamez[3], Jo Vandesompele[1,2]
[1]Ghent University, Center for Medical Genetics Ghent, Ghent, Belgium; [2]pxlence, Dendermonde, Belgium; [3]Ghent University, Biomarked, Ghent, Belgium;steve.lefever@ugent.be

15:00 **Customized Solution Hybridization Enrichment Panels: From A Single Gene To An Entire Exome- Design, Usage, And How To Increase Your Odds For A Successful Outcome**

Scott Rose
Integrated DNA Technologies, United States of America; srose@idtdna.com

15:30 **High-Throughput Single-Cell Sequencing Library Generation Using Nextera And The LabCyte ECHO 525**

Stephan Lorenz
Wellcome Trust Sanger Institute, United Kingdom; sl11@sanger.ac.uk

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

16:30 **Inferring Ribosome Dynamics From mRNA Degradome Sequencing.**

Vicent Pelechano[1], Wu Wei[2,3], Lars M Steinmetz[1,2,3]
[1]European Molecular Biology Laboratories, Genome Biology Unit, Germany; [2]Stanford Genome Technology Center, Stanford University, USA; [3]Department of Genetics, Stanford University School of Medicine; pelechan@embl.de

17:00 **Sample to Insight - analysis, visualization and interpretation of next generation sequencing and gene expression data**

Anne Arens
QIAGEN GmbH, Germany; anne.arens@qiagen.com

17:30 **High resolution NGS-based HLA-typing using in-solution targeted enrichment**

Michael Wittig[1], Jarl Andreas Anmarkrud[2,3,4], Jan Christian Kässens[5], Simon Koch[6], Michael Forster[1], Eva Ellinghaus[1], Johannes E.R. Hov[2,4,7], Sascha Sauer[8], Manfred Schimmler[5], Malte Ziemann[9], Siegfried Görg[9], Frank Jacob[6], Tom Hemming Karlsen[2,4,7], Andre Franke[1]
[1]Christian-Albrechts-University Kiel, Institute of Clinical Molecular Biology, [2]Norwegian PSC Research Center, Department of Transplantation Medicine, [3]K.G. Jebsen Inflammation Research Center, Institute of Clinical Medicine, [4]Research Institute of Internal Medicine, [5]Christian-Albrechts-University of Kiel, Department of Computer Science, [6]Muthesius Academy of Fine Arts and Design, [7]Section of Gastroenterology, Department of Transplantation Medicine, [8]Max-Planck Institute for Molecular Genetics, Berlin; [9]University of Lübeck, Institute of Transfusion Medicine, Lübeck, Germany; m.wittig@mucosa.de

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18:00 – 22:00 **Evening Poster Session**

18:00 – 22:00 **Networking Reception in Industrial Exhibition**

Tuesday 24th March 2015

Digital PCR

Tuesday, 24/Mar/2015:
8:30am - 10:30am
Session Chair:
Jim Francis Huggett
Tim Forshew

Location:
Lecture hall 14

Presentations

8:30 **Keynote Lecture:**

How could digital PCR benefit clinical analysis?

Jim Francis Huggett
LGC, United Kingdom; jim.huggett@lgcgroup.com

9:00 **Rapid and Ultra-Sensitive Single-Cell Transcript Profiling with Droplet Digital PCR (ddPCR™): Application to neural induction**

Svilen Tzonev
Digital Biology Center, Bio-Rad, United States of America; Svilen_Tzonev@bio-rad.com

9:30 **NGS and dPCR Analysis of Circulating Tumour DNA and Their Potential Uses in Cancer Patient Care**

Tim Forshew
UCL, United Kingdom; t.forshew@ucl.ac.uk

10:00 **Droplet generation for NGS library preparation and for digital PCR on the same instrument**

Sabit Delic, Manja Meggendorfer, Niroshan Nadarajah, Wolfgang Kern, Torsten Haferlach
MLL Münchner Leukämielabor GmbH, Germany; sabit.delic@mll.com

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

Non-coding RNAs 1

Tuesday, 24/Mar/2015:
11:00am - 12:30pm
Session Chair:
Jo Vandesompele
Robert Sjöback

Location:
Lecture hall 14

Presentations

11:00 **Keynote Lecture:**

Updated evaluation of quantitative miRNA expression platforms in the microRNA quality control (miRQC) study

Pieter Mestdagh, Jo Vandesompele
Ghent University / Biogazelle, Belgium (on behalf of the microRNA quality control study consortium); joke.vandesompele@ugent.be

11:30 **MicroRNA profiling using a rapid, highly sensitive qPCR panel**

Simon Baker[1], Lihan Zhou[2], Florent Chang Pi Hin[1], Ruiyang Zou[2], Heng-Phon Too[2]
[1]Bioline Reagents Ltd, United Kingdom; [2]MIRXES Pte. Ltd, Singapore; Simon.Baker@bioline.com

12:00 **Fast and flexible bioinformatics analysis of RNA seq data to provide biological insight**

Jesper Culmsee Tholstrup
EXIQON, Denmark; jet@exiqon.com

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

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

Life Technologies: Lunch-time seminars

Tuesday, 24/Mar/2015:
1:00pm - 2:00pm
Session Chair:
Thomas Schild

Location:
Lecture hall 14

Presentations

13:00 **Digital PCR analysis of tumor-specific mutations from peripheral blood in ER positive breast cancer samples**

Atocha Romero
Hospital Universitario Puerta de Hierro, Spain; atocha10@hotmail.com

13:30 **Highly sensitive amplicon-based RNA quantification by Ion Torrent Proton semiconductor sequencing.**

Ulrich Certa
Hoffmann-La Roche AG, Switzerland; ulrich.certa@roche.com

Biomarker Discovery & Circulating Nucleic Acids

Tuesday, 24/Mar/2015:
2:00pm - 6:30pm
Session Chair:
Stephen Andrew Bustin
Michael W. Pfaffl

Location:
Lecture hall 14

Presentations

14:00 **Keynote Lecture:**

Mass Spectrometry Based Draft Of The Human Proteome

Bernhard Kuster
TU München, Germany; kuster@tum.de

14:30 **CircRNA: a new class of non-coding ribonucleic acid molecules**

Stanislas Werfel
Technische Universität München, Germany; werfel@ipt.med.tum.de

15:00 **Rapid High Sensitivity Monitoring of Tumor Genetic Changes for Targeted Therapy of Cancer**

Michael Joseph Powell, Aiguo Zhang, Madhuri Ganta, Elena Peletskaya
DiaCarta, Inc., United States of America
mpowell@apollolongevity.com

15:30 **Proximity Ligation Assay for the specific detection of *Aspergillus* species**

Gemma Johnson[1], Mark Shannon[2], Christopher Thornton[3], Samir Agrawal[4], Cornelia Lass-Flörl[5], Wolfgang Mutschlechner[5], Stephen Bustin[1]
[1]Postgraduate Medical Institute, Faculty of Medical Science, Anglia Ruskin University, UK; [2]Thermo Fisher Scientific, USA; [3]School of Biosciences, University of Exeter, UK; [4]Blizard Institute of Cellular and Molecular Science, Queen Mary University, London, UK; [5]Division of Hygiene and Medical Microbiology, Innsbruck Medical University, Innsbruck, Austria; gljohnson82@gmail.com

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

16:30 **Extracellular Vesicles As Disease Biomarkers?**

Mercedes Tkach[1,2], Joanna Kowal[1,2], Clotilde Thery[1,2]
[1]Institut Curie Section recherche, Paris, France; [2]INSERM U932, Paris, France; mercedes.tkach@curie.fr

17:00 **Cell to Cell Communication via extracellular RNA and Exosomes**

Michael W Pfaffl
TUM, Germany; michael.pfaffl@wzw.tum.de

17:30 **Exosomes – A novel approach to biomarker identification**

Constanze Kindler[1], Jonathan Shaffer[2]
[1]QIAGEN GmbH, Germany; [2]QIAGEN Sciences; constanze.kindler@qiagen.com

18:00 **RT-qPCR based screening of blood-circulating microRNAs for the identification of biomarkers and novel drug targets**

Matthias Hackl[1], Sylvia Weilner[1], Susanna Skalicky[1], Rita Reynoso[2], Natalia Laufer[3], Heribert Stoiber[4], Peter Dovjak[5], Peter Pietschmann[6], Johannes Grillari[1,2]
[1]TAmiRNA GmbH, Muthgasse 18, 1190 Vienna, Austria; [2]Department of Biotechnology, University of Natural Resources and Life Sciences Vienna, Vienna, Austria;[3]CONICET, Buenos Aires, Argentina; [4]Division of Virology, Innsbruck Medical University, Innsbruck, Austria; [5]Salzkammergutklinikum Gmunden, Austria; [6]Department of Pathophysiology and Allergy Research, Medical University Vienna, Austria; matthias.hackl@tamirna.com

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

Location: **Bräustüberl Weihenstephan**
Weihenstephaner Berg 10
85354 Freising
European – Bavarian - Asian Buffet
Music & Dancing & Cocktails
www.Braeustueberl-Weihenstephan.de

BRÄUSTÜBERL Weihenstephan

Tuesday 24th March 2015

Next Generation Sequencing 2

Tuesday, 24/Mar/2015:
8:30am - 10:30am
Session Chair:
Karen E. Nelson
Michael W. Pfaffl

Location:
Lecture hall 15

Presentations

8:30 **Keynote Lecture:**

Next Generation Sequencing Applications to the study of human and animal Microbiomes.

Karen E. Nelson
J. Craig Venter Institute, United States of America; karennel@gmail.com

9:00 **DNA-based Food Authenticity and Safety Control using Advanced Barcoding by Next Generation Sequencing**

Ilka Haase, Christine Käppel, Elmar Schilling
Eurofins Genomics, Germany; ilkahaase@eurofins.com

9:30 **Integrative Analyses of Primary and Relapse Tumor Samples Identifies Genomic Changes During Cancer Progression**

Alexander Schramm
University Hospital Essen, Germany; alexander.schramm@uni-due.de

10:00 **Microbiomes via sequencing: Challenges and opportunities**

Thomas Clavel
Junior Research Group Intestinal Microbiome, TU München, Germany; thomas.clavel@tum.de

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

NGS data analysis

Tuesday, 24/Mar/2015:
11:00am - 12:30pm
Session Chair:
Robert P. Loewe
Michael W. Pfaffl

Location:
Lecture hall 15

Presentations

11:00 **Benchmarking of RNA-seq data processing pipelines using whole transcriptome qPCR expression data**

Jan Hellemans[1], Jo Vandesompele[1,2], Pieter Mestdagh[1,2]
[1]Biogazelle, Belgium; [2]CMGG, UGent, Belgium; jan.hellemans@biogazelle.com

11:30 **Chances and challenges in RNA-Seq analysis**

Matthias Scherf & Martin Seifert
Genomatix, Germany; seifert@genomatix.de

12:00 **RNAseq data analysis: getting more insight by combining it with public RNAseq experiments**

Philip Zimmermann
Nebion AG, Switzerland; phz@nebion.com

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

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

Agilent Technologies: Lunch-time seminars

Tuesday, 24/Mar/2015:
1:00pm - 2:00pm
Session Chair:
Raza Ahmed
Bernd Martin

Location:
Lecture hall 15

Presentations

13:00 **High Resolution Melting – How To Produce Highly Reproducible Measurements**

Robert P. Loewe
GeneWake GmbH, Germany; robert.loewe@genewake.com

13:30 **Total Confidence qPCR – The New AriaMX Real-Time PCR System**

Bernd Martin
Agilent Technologies, Germany; bernd.martin@agilent.com

Microgenomics & Single-Cell Diagnostics

Tuesday, 24/Mar/2015:
2:00pm - 6:30pm
Session Chair:
Anders Ståhlberg
Mikael Kubista

Location:
Lecture hall 15

Presentations

14:00 **Keynote Lecture:**

Expression Profiling of Circulating Tumor Cells: a Prognostic and Predictive Biomarker in Cancer.

Mikael Kubista[1,2], Robert Sjöback[1], Marie Jindrichova[2], Eva Rohlova[1], Vendula Novosadová[1,2], Siegfrid Hauch[3], Katarina Kolostova[4], Bahriye Aktas[5], Mitra Tewes[5], Maren Bredemeier.[5], Sabine Kasimir-Bauer[5]
[1]TATAA Biocenter, Sweden; [2]Institute of Biotechnology, CAS, Czech Republic; [3]AdnaGen; [4]Charles University; [5]University Hospital Essen; mikael.kubista@tataa.com

14:30 **Revolutionizing our view of the genome and transcriptome through innovative sequencing workflows**

Dave Ruff, Marc Unger, Jay West, Richard Fekete, Robert Jones
Fluidigm Corporation, South San Francisco, CA USA. dave.ruff@fluidigm.com

15:00 **From Cultures to Results in One Day: A New Workflow for Accurate and Reproducible Large Scale qPCR Expression Analysis from Limited Samples in Stem Cell Research**

Mark Kibschull[1], Stephen Lye[1,2,3], Steven Okino[4], Haya Sarras[5]
[1]Lunenfeld-Tanenbaum Research Institute, Mount Sinai Hospital, Toronto, Canada; [2]Departments of OBS/GYN, Physiology, and Medicine, University of Toronto, Toronto, Canada; [3]Fraser Mustard Institute for Human Development, University of Toronto, Toronto, Canada; [4]Gene Expression Division, Life Science Group, Bio-Rad Laboratories, Hercules, United States; [5]Gene Expression Division, Life Science Group,

Bio-Rad Laboratories, Mississauga, Canada; kibschull@lunenfeld.ca

15:30 **Feasibility of a workflow for the molecular characterization of single Circulating Tumor Cells by Next Generation Sequencing.**

Francesca Salvianti[1], Giada Rotunno[2], Francesca Galardi[3], Francesca De Luca[3], Marta Pestrin[3], Alessandro Maria Vannucchi[2], Angelo Di Leo[3], Mario Pazzagli[1],Pamela Pinzani[1]
[1]Department of Clinical, Experimental and Biomedical Sciences, University of Florence, Florence Italy.; [2]Department of Clinical and Experimental Medicine, University of Florence, Florence Italy; [3]Sandro Pitigliani Medical Oncology Department, Hospital of Prato, Istituto Toscano Tumori, Prato, Italy; p.pinzani@dfc.unifi.it

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

16:30 **Single-cell analysis of Myxoid liposarcoma reveals novel subpopulations and dysfunctional cell cycle regulation**

Anders Ståhlberg
University of Gothenburg, Sweden; anders.stahlberg@gu.se

17:00 **SMARTer Solutions for Low-Input Transcriptome Sequencing …and more !**

François-Xavier Sicot
Takara Clontech, Francois-Xavier.Sicot@takara-clontech.eu

17:30 **G&T-seq: Separation and parallel sequencing of the genomes and transcriptomes of single cells**

Iain Macaulay[1], Wilfried Haerty[2], Parveen Kumar[3], Yang Li[2], Tim Hu[2], Mabel Teng[1], Niels van der Aa[3], Paul Coupland[1], Chris Ponting[1,2], Thierry Voet[1,3]
[1]Sanger Institute, United Kingdom; [2]MRC Functional Genomics Unit, Oxford; [3]KU Leuven; im2@sanger.ac.uk

18:00 **Intracellular molecular gradients within Xenopus laevis oocytes prepare the cell for asymmetric division**

Radek Sindelka, Monika Sidova, Mikael Kubista
IBT AS CR, Czech Republic; sindelka@ibt.cas.cz

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

Location: Bräustüberl Weihenstephan
Weihenstephaner Berg 10
85354 Freising
European – Bavarian - Asian Buffet
Music & Dancing & Cocktails
www.Braeustueberl-Weihenstephan.de

Wednesday 25th March 2015

Optimisation, MIQE & QC strategies in qPCR

Wednesday, 25/Mar/2015:
8:30am - 12:50pm
Session Chair:
Tania Nolan
Afif M. Abdel Nour

Location:
Lecture hall 14

Presentations

8:30 **Keynote Lecture:**

Variability of the Reverse Transcription Step: Practical Implications

Stephen Bustin[2,1], Harvinder Dhillon[2], Sara Kirvell[2], Christina Greenwood[2], Michael Parker[2], Gregory Shipley[4,1], Tania Nolan[1,3]
[1]The Gene Team, United Kingdom; [2]Postgraduate Medical Institute, Anglia Ruskin University, Cambridge, UK; [3]Institute of Population Health, University of Manchester, Manchester UK; [4]Shipley Consulting, LLC, Austin, Texas, USA; tania.nolan@geneteamconsultants.com

9:00 **qScript XLT cDNA SuperMix: overcoming the common pitfalls of cDNA synthesis as applied to relative quantification and RT-qPCR.**

David Mark Schuster, Yun Feng
Quanta BioSciences, United States of America; dave.schuster@quantabio.com

9:30 **New tools for quality assessment of RNA in Molecular Diagnostics**

Jens Björkman[1], David Švec[1,2], Robert Sjöback[1], Emelie Lott[1], Mikael Kubista[1,2]
[1]TATAA Biocenter AB, Sweden; [2]Laboratory of Gene Expression, Institute of Biotechnology, Academy of Sciences of the Czech Republic, Prague, Czech Republic;jens.bjorkman@tataa.com

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

10:30 **ThermaStop™: Properties and Benefits**

Alexandra Isabel Over[1], Lawrence Wangh[2]
[1]Biosearch Technologies, United States of America; [2]Brandeis University, USA; AlexOver@brandeis.edu

11:00 **Engineered DNA Polymerases**

Ramon Kranaster
myPOLS Biotec UG (haftungsbeschränkt), Universitätsstr. 10, 78457 Konstanz, Germany, www.mypols-biotec.com, T +49(0)7531 884654; ramon.kranaster@mypols.de

11:30 **Reducing time and error rates by automating qPCR workflows**

Matjaž Hren[1], Kristina Gruden[2], Klemen Zupančič[1], Nataša Mehle[2], Manca Pirc[1,2], Urška Čepin[1], Maja Ravnikar[2]
[1]BioSistemika LLC, Slovenia; [2]National Institute of Biology, Slovenia; matjaz.hren@biosistemika.com

12:00 **Middleware Solutions To Improve Molecular Diagnostics; Connectivity, Quality Control And Lean Processes**

HGM Niesters[1], F Klein[1], J Poodt[2]
[1]Department of Medical Microbiology, Division of Clinical Virology, UMC Groningen; [2]LabHelp, Bladel, The Netherlands; h.g.m.niesters@umcg.nl

12:30 **Digital publication: how making the MIQE guidelines easier to follow.**

Afif M. Abdel Nour[1], Michael W. Pfaffl[2]
[1]Bio-Rad, United Arab Emirates; [2]TUM Physiology, Freising, Germany; iBook@bioMCC.com

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

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

Non-coding RNAs 2

Wednesday, 25/Mar/2015:
2:00pm - 4:30pm
Session Chair:
Pieter Mestdagh

Location:
Lecture hall 14

Presentations

14:00 **Keynote Lecture:**

Decoding lncRNA functions using high-throughput pathway perturbation.

Pieter Mestdagh, Jan Hellemans, Ariane De Ganck, Jo Vandesompele
Biogazelle, Belgium; pieter.mestdagh@biogazelle.com

14:30 **MiRNA Profiling In Tumor Tissue, Body Fluids And Exosomes - A Combinational Techniques Approach Of NGS And QPCR.**

Robert P. Loewe
GeneWake GmbH,
Germany; robert.loewe@genewake.com

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

15:30 **Development and Optimisation of PCR Assays to Analyse MicroRNAs and their Target Genes**

David Arthur Simpson
Queen's University Belfast, United Kingdom; david.simpson@qub.ac.uk

16:00 **Improved Small RNA Library Preparation Workflow for Next Generation Sequencing**

Sabrina Shore, Jordana Henderson, Anton McCaffrey, Gerald Zon, Richard Hogrefe
TriLink Biotechnologies, United States of America; sshore@trilinkbiotech.com

Wednesday 25th March 2015

qPCR BioStatistics & BioInformatics

Wednesday, 25/Mar/2015:
8:30am - 12:30pm
Session Chair:
Jan Ruijter
Stefan Rödiger

Location:
Lecture hall 15

Presentations

8:30 **Removal of Between-Plate Variation in qPCR with Factor Correction: Completion of the Analysis Pipeline Supported by RDML.**

Jan Ruijter[1], Jan Hellemans[2], Adrian Ruiz-Villalba[1], Maurice van den Hoff[1], Andreas Untergasser[3]
[1]Academic Medical Center, the Netherlands; [2]Biogazelle, Belgium; [3]Heidelberg University, Heidelberg, Germany; j.m.ruijter@amc.uva.nl

9:00 **Unexpected System-specific Periodicity In Quantitative Real-Time Polymerase Chain Reaction Data And Its Impact On Quantification**

Andrej-Nikolai Spiess[1], Stefan Rödiger[2], Thomas Volksdorf[3], Joel Tellinghuisen[4]
[1]Department of Andrology, University Hospital Hamburg-Eppendorf, Germany; [2]Faculty of Natural Sciences, BTU Cottbus – Senftenberg, Cottbus, Germany; [3]Department of Dermatology, University Hospital Hamburg-Eppendorf, Germany; [4]Department of Chemistry, Vanderbilt University, Nashville, Tennessee, USA; a.spiess@uke.de

9:30 **The PrimerBank database: an analysis of high-throughput primer validation.**

Athanasia Spandidos[1,2,3], Xiaowei Wang[1,2,4], Huajun Wang[1,2], Brian Seed[1,2]
[1]Center for Computational and Integrative Biology, Massachusetts General Hospital, Boston, MA; [2]Department of Genetics, Harvard Medical School; [3]Current address: 1st Department of Pathology, National and Kapodistrian University of Athens, Athens, Greece.; [4]Current address: Division of Bioinformatics and Outcomes Research, Department of Radiation Oncology, Washington University School of Medicine, St Louis, MO.; a.spandidos@gmail.com

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

10:30 **Occurrence of unexpected PCR artefacts warrants thorough quality control**

Adrián Ruiz-Vilalba[1], Bep van Pelt-Verkuil[2], Quinn Gunst[1], Maurice van den Hoff[1], Jan Ruijter[1]
[1]Department of Anatomy, Embryology and Physiology, Academic Medical Centre (AMC), Amsterdam, The Netherlands; [2]Department of Innovative Molecular Diagnostics, University of Applied Sciences, Leiden, the Netherlands; a.ruizvillalba@amc.nl

11:00 **Impact of Smoothing on Parameter Estimation in Quantitative DNA Amplification Experiments**

Stefan Rödiger[1], Andrej-Nikolai Spiess[2], Michał Burdukiewicz[3]
[1]BTU Cottbus - Senftenberg, Senftenberg, Germany; [2]University Medical Center Hamburg-Eppendorf, Hamburg, Germany; [3]University of Wroclaw, Wroclaw, Poland;Stefan.Roediger@b-tu.de

11:30 **RDML qPCR Data Format - Ready For The Next Level?**

Andreas Untergasser[1], Steve Lefever[2], Jasper Anckaert[2], Jan M Ruijter[3], Jan Hellemans[4], Jo Vandesompele[2,4]
[1]University of Heidelberg, Heidelberg, Germany; [2]Ghent University, Ghent, Belgium; [3]Academic Medical Center, Amsterdam, The Netherlands; [4]Biogazelle, Zwijnaarde, Belgium; andreas@untergasser.de

12:00 **RDML Consortium Meeting**

Andreas Untergasser and coworkers
On behalf of the RDML consortium

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

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

BDQ Editors Board Meeting

Wednesday, 25/Mar/2015:
12:30pm – 1:30pm
Session Chair:
Stephen Andrew Bustin
Frauke Münzel

Location:
Lecture hall 15

BDQ Poster Awards

Wednesday, 25/Mar/2015:
1:45pm - 2:00pm
Session Chair:
Stephen Andrew Bustin
Frauke Münzel

Location:
Lecture hall 15

Molecular Diagnostics in Agriculture, Veterinary Medicine, Food & Environmental Science

Wednesday, 25/Mar/2015:
2:00pm - 4:30pm
Session Chair:
Irmgard Riedmaier-Sprenzel
Michael W. Pfaffl

Location:
Lecture hall 15

Presentations

14:00 **Comparison of Different RNA Sources to Examine the Lactating Bovine Mammary Gland Transcriptome using RNA-Sequencing**

Angela Canovas[1], Claudia Bevilacqua[2], Gonzalo Rincon[1], Pauline Brenaut[2], Alma Islas-Trejo[1], Russell C. Hovey[1], Marion Boutinaud[3], Caroline Morgenthaler[2], Monica K. VanKlompenberg[1], Juan F. Medrano[1], Patrice D. Martin[2]
[1]Department of Animal Science, University of California-Davis, One Shields Avenue, Davis, 95616, CA, USA; [2]Institut National de la Recherche Agronomique, UMR 1313 Génétique animale et Biologie intégrative, F-78350 Jouy-en-Josas, France; [3]INRA, AGROCAMPUS OUEST, UMR1348 PEGASE, F-35590 Saint-Gilles, France;patrice.martin@jouy.inra.fr

14:30 **Optimized library preparation for sequencing of whole bacterial genomes and low density microbiota by Illumina-based NGS**

Mareike Wenning, Christopher Huptas, Manuela Schreiner, Siegfried Scherer
Lehrstuhl für Mikrobielle Ökologie, Zentralinstitut für Ernährungs- und Lebensmittelforschung, Technische Universität München; mareike.wenning@wzw.tum.de

15:00 **Transcriptomic Biomarkers in Food Safety: RNA Biomarkers against the abuse of growth promoters**

Irmgard Riedmaier-Sprenzel, Melanie Spornraft, Michael, W. Pfaffl
TUM Physiology,
Germany; irmgard.riedmaier@wzw.tum.de

15:30 **Challenges in Detection of Genetically Modified Organisms**

Mojca Milavec, David Dobnik, Jana Žel
National Institute of Biology,
Slovenia; mojca.milavec@nib.si

16:00 **A Transcriptomic Approach To Ascertain Insights Into The Etiology Of Brown Trout Syndrome**

Marc Young[1], Jens-Eike Taeubert[1], Juergen Geist[2], Michael W. Pfaffl[3], Ralph Kuehn[1]
[1]Unit of Molecular Zoology, Chair of Zoology, Department of Animal Science, TUM; [2]Aquatic Systems Biology Unit, Department of Ecology and Ecosystem Management, TUM; [3]Physiology Weihenstephan, Department of Animal Sciences, TUM;
marc.young@tum.de

Closing of the Symposium Lecture hall HS 14

16:30 **Closing of the Symposium & Farewell**
Michael W. Pfaffl
Sylvia Pfaffl

Workshops: Thursday 26th March & Friday 27th March 2015

The workshops are aimed at giving participants a deep and objective understanding of real-time quantitative PCR, Next Generation Sequencing, biostatistics, expression profiling, digital-PCR, and its applications. The courses are intended for academic or industrial persons considering working with qPCR and/or NGS or scientists currently working with thes technologies seeking a deeper understanding. All qPCR workshops offer extensive hands-on training by qPCR or NGS expertsin the field.

The qPCR workshops on 26th and 27th March (9 am – 5 pm) are hosted by TATAA Biocenter (www.TATAA.com) or Bio-Rad (www.Bio-Rad.com). The NGS data analysis workshop on 26th and 27th March (9 am – 5 pm) are hosted by Genomatix (www.Genomatix.com) and Qiagen (www.Qiagen.com).

Workshop topics:

- **Basic real-time qPCR Application Workshop** (2-days)
  hosted by TATAA Biocenter (Seminar room – S3)
- **qPCR Data Analysis Workflow: from instrument data to interpretation** (2-days)
  hosted by TATAA Biocenter (Computer seminar room – PU26)
- **digital PCR** (2-days)
  hosted by Bio-Rad (Seminar room – S1)
- **NGS data analysis workshop** (2-days)
  hosted by Genomatix (Computer seminar room – PU26A - GIS room)
- **"Sample to Insight" Analyzing and interpreting the biological meaning in NGS data** (2-days)
  hosted by Qiagen (Computer seminar room – HU34A)

More info about the workshops http://workshops.qPCR-MGS-2015.net

Talk Session Abstracts

Advanced Molecular Diagnostics 1

Monday, 23/Mar/2015:
10:30am - 12:30pm
Session Chair:
Stephen Andrew Bustin
Michael W. Pfaffl

Location:
Lecture hall 14

Presentations

PCR in less than 30 seconds: Efficient, specific amplification with increased primer and polymerase.

Jared S Farrar[1], Carl T Wittwer[2]
[1]Virginia Commonwealth University; [2]University of Utah; carl.wittwer@path.utah.edu

BACKGROUND: PCR is a key technology in molecular biology and diagnostics that typically amplifies and quantifies specific DNA fragments in about an hour. However, the kinetic limits of PCR are unknown.

METHODS: We developed prototype instruments to temperature cycle 1- to 5-µL samples in 0.4–2.0 s at annealing/extension temperatures of 62°C–76°C and denaturation temperatures of 85°C–92°C. Primer and polymerase concentrations were increased 10- to 20-fold above typical concentrations to match the kinetics of primer annealing and polymerase extension to the faster temperature cycling. We assessed analytical specificity and yield on agarose gels and by high-resolution melting analysis. Amplification efficiency and analytical sensitivity were demonstrated by real-time optical monitoring.

RESULTS: Using single-copy genes from human genomic DNA, we amplified 45- to 102-bp targets in 15–60 s. Agarose gels showed bright single bands at the expected size, and high-resolution melting curves revealed single products without using any “hot start” technique. Amplification efficiencies were 91.7%–95.8% by use of 0.8- to 1.9-s cycles with single-molecule sensitivity. A 60-bp genomic target was amplified in 14.7 s by use of 35 cycles.

CONCLUSIONS: The time required for PCR is inversely related to the concentration of critical reactants. By increasing primer and polymerase concentrations 10- to 20-fold with temperature cycles of 0.4–2.0 s, efficient (>90%), specific, high-yield PCR from human DNA is possible in <15 s. Extreme PCR demonstrates the feasibility of while-you-wait testing for infectious disease, forensics, and any application where immediate results may be critical.

Laser-driven PCR for ultrafast DNA detection

Lars Ullerich, Federico Bürsgens, Joachim Stehr
GNA Biosolutions GmbH, Germany; ullerich@gna-bio.com

GNA has developed Laser PCR, for ultra-fast molecular diagnostics. This method is based on laser-heated nanoparticles. In comparison to conventional DNA amplification, we do not heat up and cool down the entire sample solution, because the nanoparticles that are added to the solution heat up immediately upon irradiation by the laser, and cool down just as quickly.

We have established an assay of three minutes for a fluoresence-free, Real-Time Laser PCR.

Digital PLA

Harvinder Singh Dhillon[1], Christina Greenwood[1], Gemma Johnson[1], Mark Shannon[2], Doug Roberts[3], Stephen Bustin[1]
[1]Anglia Ruskin University, Chelmsford, Essex, United Kingdom; [2]Thermo Fisher Scientific, Sunnyvale, CA, USA; [3]Formulatrix, Bedford, MA, USA; stephen.bustin@anglia.ac.uk

The proximity ligation assay (PLA) is an advanced quantitative, sensitive and relatively inexpensive immunoassay technology that can be adapted for several assay formats, including protein detection by PCR. The binding of two antibodies, which are coupled to different, non-complementary oligonucleotides, to their target protein facilitates the enzymatic ligation of the oligonucleotides and the detection of the resulting amplicon by real-time quantitative PCR (qPCR) acts as a surrogate marker for the protein of interest. Hence PLA has potential as a clinically relevant diagnostic tool for the detection of fungal and bacterial pathogens, where PCR cannot readily identify viable, infectious or non-pathogenic strains. We shall discuss the design of innovative PLAs that use single monoclonal antibodies for the detection of *Clostridium difficile*, an important health care-associated pathogen. We also report for the first time the use of digital PCR to generate a robust dualplex digital PLA (dPLA) targeting the two major virulence factors TcdA and B. We conclude that PLA and dPLA have potential as new diagnostic applications for the detection of pathogens where nucleic acid based tests are inconclusive proof of infection. Importantly, since it is not always necessary to use two different antibodies, the pool of potential antibodies useful for PLA diagnostic assays is vastly enhanced.

New advances in sequencing-based technology in Clinical Application and Molecular Diagnosis

Fang Chen, Ya Gao, Hui Jiang, Yanyan Zhang
BGI-Shenzhen, China, People's Republic of; fangchen@genomics.cn

Massively paralell sequencing have been applied in seveal fields in clinical setting in the past few years. Most research and application focus on genetic diseases and cancer. Since 2011, the sequencing-based noninvasive prenatal testing has been successfully intergrated into current prenatal screening and dianosis workflows in the clinical practice and brings a revolutionarily change in healthcare-related industrials. With the rapidly development of ultra high-throughput and faster sequencing technologies and increasing mature regulation and standarlization on relative commercial kits or products, it will be very possible to expand quickly to more clinical fields and intergrate deeply into current medical service workflows.

Advanced Molecular Diagnostics 2

Monday, 23/Mar/2015:
2:00pm - 6:00pm
Session Chair:
Justin O'Grady
Mikael Kubista

Location:
Lecture hall 14

Presentations

Pathogen DNA enrichment strategies for nanopore sequencing based infectious diseases diagnostics.

Justin O'Grady
University of East Anglia, United Kingdom; justin.ogrady@uea.ac.uk

The infectious diseases which are most important to diagnose rapidly are those which are life threatening. These include sepsis and pneumonia, which have complex aetiology and which require the recognition of pathogens within challenging sample matrices. The “gold standard” culture techniques are labour intensive, have long turn-around times and offer poor clinical sensitivity. Culture takes at least two days: one to grow the bacteria and then, at best, one to identify pathogens and test their antimicrobial susceptibility. Meanwhile the patient is treated empirically, which often results in inappropriate treatment. Nucleic acid amplification tests (NAATs) are a step in the right direction, providing results within hours, but are limited by the range of pathogens they can detect.

A paradigm shift in diagnostics technology is required, to allow the development of a universal diagnostic which can detect any pathogen, known or unknown. We are developing the first culture independent non-targeted (i.e. no specific amplification or capture) next generation sequencing (NGS) based methods for the routine diagnosis of clinical syndromes such as sepsis and UTIs. NGS based ID diagnostics represents a disruptive advance in the field, combining the speed of NAATs with comprehensiveness beyond that of culture (capable of detecting bacteria, viruses and fungi).

One of the biggest challenges to successfully applying NGS in medical microbiology is the vast quantity of host vs pathogen nucleic acid present in clinical samples such as blood. We are combining novel pathogen DNA enrichment techniques with the latest nanopore NGS technologies (Oxford Nanopore MinION device) to make this possible.

I will describe our pathogen DNA enrichment strategies and present data from our projects on the NGS based diagnosis of blood stream and urinary tract infections. I will also discuss some of our other applications of MinION sequencing technology and present our most recent performance data.

Differentiation of Identical Twins using Next Generation Sequencing

Burkhard Rolf
Eurofins, Germany; BurkhardRolf@eurofins.com

Monozygotic (MZ) twins are considered being genetically identical, therefore they cannot be differentiated using standard forensic DNA testing. Here we describe how identification of extremely rare mutations by ultra-deep next generation sequencing can solve such cases. We sequenced DNA from sperm samples of two twins and from a blood sample of the child of one twin. Bioinformatics analysis revealed five Single Nucleotide Polymorphisms (SNPs) present in the twin father and the child, but not in the twin uncle. The SNPs were confirmed by classical Sanger sequencing. The method provides a solution to solve paternity and forensic cases involving monozygotic twins as alleged fathers or originators of DNA traces.

Mobile applications for qPCR

Jo-Ann Stanton
Ubiquitome, New Zealand; jo.stanton@anatomy.otago.ac.nz

Since its invention in the 1980s PCR has become a cornerstone technology for life science research and is transforming molecular diagnostics. But it is only recently that PCR has emerged from the traditional laboratory to impact health and environmental screening programs. True escape from the laboratory potentially changes medical practice, proscription drug use and disease surveillance. Over the past 6 years we have developed technology to perform qPCR in resource poor settings. The device, called the Freedom4, is a battery-operated, laser optic, WiFi enabled instrument that performs qPCR and delivers comparable results to larger in-laboratory instruments. Independent testing has been carried out on a number of human pathogens and clinical samples, including *E. coli* STEC, influenza and norovirus. Current work with groups from across the world focuses on non-invasive genetic analysis of endangered species, environmental screening and disease surveillance. This work demonstrates the potential impact mobile applications for qPCR could deliver.

"NanoString - The Next Step After NGS"

Michael Rhodes
NanoString Technologies; mrhodes@nanostring.com

TRANSLATIONAL MEDICINE -- Moving from NGS results to Gene Signature

Converting insights from data-dense, next-gen sequencing and expression profiling approaches into “clinical-strength” multi-gene assays remains a challenge. Distinctive technologies developed by Seattle, WA-based NanoString Technologies (founded in 2003) which provide simple, automated, digital profiling of single molecules will be described. The methodology is based on an optical digital molecular barcoding technology invented at the Institute for Systems Biology (ISB) under the direction of Dr. Leroy Hood (Nature Biotech (2008) 26:317-25).

Using NanoString technology, up to 800 distinct nucleic-acid targets can be digitally counted in samples that range from single cells (10 pg input RNA) to 30-yr-old formalin-fixed, paraffin-embedded tumor specimens.

NanoString recently introduced a new chemistry called “nCounter Elements™” that allows individual labs to create their own custom codesets using ordinary oligonucleotides (purchasable from any oligo-vendor). NanoString optical-barcode reagents (sold as a General Purpose Reagent) interact with 3rd party sourced generic oligonucleotides and allow individual labs to quickly design and implement their own assays that can be utilized for research purposes or for Laboratory Developed Tests.

NanoString recently received a CE Mark for its breast cancer gene expression test, allowing NanoString to offer the test on its nCounter system in the European Union and other countries that recognize the CE Mark. Nanostring has just received FDA 510(k) Clearance for its Prosigna™ Breast Cancer Prognostic Gene Signature Assay. Additional research areas in biology (single-cell, miRNA, CNV, gene-fusions, plant studies, non-human studies) and clinical opportunities in medicine (prognostic and predictive genomics) will be explored during the presentation.

Optimized Targeted NGS With Multiplex Competitive PCR Amplicon Libraries for Reliable Diagnostic Testing of Cancer FFPE Samples

Jiyoun Yeo[2], Erin Crawford[2], Tom Morrison[1], Xiaolu Zhang[2], Luke Stewart[3], James Willey[1,2]

[1]Accugenomics, Inc., United States of America; [2]University of Toledo College of Medicine; [3]Fluidigm, Inc.; james.willey2@utoledo.edu

Background: A recently described targeted NGS method using multiplex competitive PCR amplicon libraries (Blomquist et al, PLOS one, 2013) was used to quantify genes comprised by the Lung Cancer Diagnostic Test (LCDT), a three gene biomarker (MYC x E2F1/p21) intended to augment accuracy of lung cancer diagnosis in transthoracic fine needle aspirate (FNA) cell block FFPE samples. The results from targeted NGS were compared to those from qPCR and revealed excellent inter-platform reproducibility (R^2= 0.99; slope = 0.95). In this method a) due to consumption of primers for abundantly expressed genes in earlier PCR cycles, there is normalization (convergence toward equivalent representation) of PCR products for lowly and highly expressed genes, and b) each target is measured relative to a known number of synthetic internal standard molecules which controls for variation in loading and/or amplification efficiency. In an effort to increase throughput, reduce reagent consumption, and further promote normalization through increased PCR efficiency of primers that perform poorly in multiplex, we implemented this method on the Fluidigm Access Array System™.

Methods: A normal bronchial epithelial cell cDNA sample was mixed with an internal standard (IS) mixture comprising 6,000 molecules of IS for each of 70 respective transcript targets. After amplification with a mixture of primers targeting each of the targets we split the product into two aliquots. One aliquot was barcoded for sequencing (Library 1) and the second was amplified in a second round on the Access Array System with only one or two targets per reaction, products re-combined into a single library and barcoded (Library 2). Platform adaptors were added, and Libraries 1 and 2 were mixed then analyzed on Illumina HiSeq™.

Results: Of 70 gene targets, 32 demonstrated at least 4-fold and up to 6,000-fold increase in target sequence depth. Differences in loading and product amplification led to widely variant sequencing depth between the two libraries for each assay. However, measured relative to the known number of respective internal standard molecules (6,000 copies), there was high inter-library correspondence for each assay (R2=0.95 ; Slope = 0.95). Further, supplemental amplification on the Access Array System better normalized amplicon yield with a 2-log reduction in coverage breadth.

Conclusions: Generation of competitive PCR amplification libraries on the Access Array System a) increased sequence yield for most targets, thereby reducing potential stochastic sampling effects, and b) promoted normalization , leading to reduced consumption of sequencing space/sample. In addition, measurement of each target relative to a known number of internal standard molecules controlled variation resulting from low sample loading and/or inefficient amplification. We conclude that this is an optimized strategy for clinical molecular diagnostic testing, including in FFPE samples.

Whole Exome Sequencing for Detection of Genetic Variants to Predict Chronic Lung Allograft Dysfunction

Markus Schmitt

GATC Biotech AG, Germany; m.schmitt@gatc-biotech.com

Schmitt M, Pison C, Wahl B, Magnan A, Royer PJ, Botturi K, Sève M, Roux A, Reynaud Gaubert M, Kessler R, Dromer C, Mussot S, Tissot A, Mal H, Guillemain R, Mornex J-F, Dahan M, Benden C, Koutsokera A, Auffray C, Marsland BJ, and Nicod LP on behalf of the SysClad consortium

SysCLAD, an European union-funded project under the FP-7, aims to identify biomarkers and personalized signatures in recipients of lung transplants to predict Chronic Lung Allograft Dysfunction (CLAD) including its subtypes Bronchiolitis Obliterans Syndrome (BOS) and Restrictive Allograft Syndrome (RAS).

A study containing DNA from 64 stable lung transplant recipients and 56 patients with diagnosed BOS (n=35) and RAS (n=21) was carried out. Exome sequencing was performed using an improved post capture enrichment protocol. All libraries were sequenced using 100 bp paired-end sequencing by synthesis technology with approximately 70 - 100 million reads. The coverage was on average between 90 - 120x per sample. Genetic variants were determined by in-house analysis pipelines. A commercial software was used to compare the relative distribution of variants between various combinations of the three designated phenotypic groups: stable, BOS and RAS. Identification of variant combinations predominant within the respective patient group was conducted by processing the resulting data in a newly developed analysis-pipeline. A median number of about 5 to 10 of such distinguishing variants were present per patient in each of the groups.

The results presented will be utilized in a panel comprising several of such variants that could identify, and possible predict, lung transplant recipients at higher risk of suffering from CLAD. A diagnostic tool with such a high sensitivity and specificity could help to reduce the number of lung transplant rejections in future.

Single Cell Analysis of Nuclear Receptor Transcription

Michael Mancini

Baylor College of Medicine, United States of America; mancini@bcm.edu

The main focus of our research has been to identify, characterize and quantify mechanistic steps of steroid nuclear receptor action at the single cell level by using state-of-the-art microscopy and bioinformatics-based approaches. To this end, we created stable cell lines and automated image analysis routines that facilitate multi-parametric small molecule, RNAi and endocrine disruptor screens to identify effectors of estrogen and androgen receptors, and also for use in primary hybridoma screening for imaging-quality monoclonal antibodies (mAbs) to receptors/coregulators. Increasingly, these studies are shifting to analysis of endogenous gene expression through multiplex mRNA FISH coupled with immunofluorescence using compatible custom-screened mAbs, and a novel, low-mag/high-resolution high throughput microscopy platform (*e.g.,* StellarVision™). These efforts have led to the creation of biological response fingerprints that identify mechanistic and phenotypic changes in biosensor and native cell lines in response to various treatments. Screens have identified novel, disease relevant estrogen receptor coregulators (*i.e.*, UBR5), receptor-specific EDCs (Bisphenol A analogs), and several repurposed drugs for use in advanced prostate cancer cell models; moreover, novel multiplex immunization and screening strategies led to generation of imaging-quality mAbs, and 'shotgun' mAb panels to tumor resistance. Collectively, these new imaging-based approaches are leading towards an improved understanding of steroid receptor and coregulator action in prostate and breast cancer models.

Next Generation Sequencing 1

Monday, 23/Mar/2015:
2:00pm - 6:00pm
Session Chair:
Jan Hellemans
Steve Lefever

Location:
Lecture hall 15

Presentations

Next-Generation RNA-Seq

Gary Schroth
Illumina, United States of America; gschroth@illumina.com

A well done RNA-Seq experiment can provide the most comprehensive, accurate and unbiased way to study gene expression, alternative splicing, RNA variation and RNA structure. The past few years have seen amazing technological advances that have led to a wide range of improvements in the RNA-Seq experimental process. Next-Generation RNA-Seq studies are unbiased, accurate, and sensitive, yet work well with low amounts of total RNA (even if the RNA is highly degraded or comes from FFPE samples). These new methods are also less expensive, require less hands-on-time, and are easier to perform than ever before. Finally the data analysis bottleneck associated with RNA-Seq studies has been completely removed with the advent of pipelines that take full advantage of massively parallel cloud computing resources. In this talk I will review the state-of-the-art in RNA-Seq experimental design, library prep, sequencing and data analysis.

Targeted resequencing and variant validation using pxlence PCR assays

Steve Lefever[1,2], Frauke Coppieters[1,2], Daisy Flamez[3], Jo Vandesompele[1,2]
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Targeted resequencing has become an important application in clinical diagnostics. A wide range of target enrichment approaches are available, enabling the customer to focus on regions of interest. This not only reduces sequencing costs per sample but also facilitates downstream data analysis considerably. Due to its flexibility in design, high sensitivity and specificity, the polymerase chain reaction (PCR) is particularly well suited as enrichment strategy.

We developed and validated a primer design tool to generate one million PCR assays for both fresh frozen and formalin fixed paraffin embedded (FFPE) samples, covering over 99% of the human exome. Assays were designed to generate equimolar and specific amplification using uniform PCR conditions. Several proof-of-concept studies using the pxlence assays have been published. NGS gene panels were developed for congenital blindness (16 genes), deafness (15 genes) and various cancer types (16 genes). Uniform sequencing coverage has been achieved using different library preparations and sequencing instruments (GS FLX, Roche; GAII, MiSeq, Illumina). To date, the Center for Medical Genetics in Ghent uses the pxlence assays in a high-throughput singleplex enrichment workflow to replace Sanger sequencing-based diagnostic tests with NGS.

Due to the excellent performance of both the design pipeline and the generated primer pairs, a spin-off called pxlence (pronounced 'pixellence') was recently founded. Its short-term goal is to provide customers easy access to the predesigned assays, enabling them to enrich any exonic region or confirm any variant of interest through either NGS or Sanger sequencing. More information is available at www.pxlence.com

Customized Solution Hybridization Enrichment Panels: From A Single Gene To An Entire Exome- Design, Usage, And How To Increase Your Odds For A Successful Outcome

Scott Rose
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While Next Generation Sequencing technology has matured to the point that entire genomes can be sequenced at an improved cost and shorter time period, there is still a very strong demand for the ability to carry out focused sequencing runs with multiple samples completed in a narrow time window. One way to accomplish this is to use custom or pre-developed and validated solution hybridization enrichment panels to selectively target only regions of interest. This way, valuable NGS machine reads are devoted to generating the depth of coverage needed, and increasing the number of samples that can be simultaneously run. This talk will cover the technical issues facing a researcher in using any hybridization based enrichment panel (custom or pre-designed), from initial design, key determinants in setting up a rapid 4 hour hybridization reaction, and how to simultaneously enrich and capture multiple libraries. Based on recent improvements in the field of hybridization enrichment, data will also be shown on what is currently achievable. In addition, practical solutions for improving or altering existing panels without having to reorder a whole new panel will be described.

High-Throughput Single-Cell Sequencing Library Generation Using Nextera And The LabCyte ECHO 525

Stephan Lorenz
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The advent of single-cell sequencing technologies enabled a new era of biological research. Researchers aim to examine 1,000s of cells to understand the composition and function of cell populations and how individual cells contribute to the greater picture of tissue and organ function, development and disease. Delivering experiments on such scale is a challenging task. Manual execution of the required protocols is laborious and today's off-the-shelf microfluidic platforms offer limited throughput. By using a combination of flow cytometry, high-speed dispensers, traditional liquid handlers and game-changing acoustic dispensing technology, we established a pipeline that delivers thousands of high-quality single-cell genomes and transcriptomes per day with a turnaround time of 2 days. Economic feasibility was achieved by minimising all assay volumes, in particular library preparation with the Nextera protocol. A conventional Nextera tagmentation and NGS library generation reaction is performed in a 50µl reaction that costs £70. By using acoustic dispensing devices, such as the ECHO 525, a scale-down by 50 – 100x can be achieved with minor modifications of the protocol, leading to significant cost reduction without sacrificing data quality. Furthermore, we demonstrate the superiority of contact-free liquid transfers in molecular biology applications with regard to reproducibility and contamination prevention. Finally, we demonstrate how acoustic dispensing can be integrated with other technologies, such as multicolour flow cytometry, to deliver next-generation experiments.

Inferring Ribosome Dynamics From mRNA Degradome Sequencing.

Vicent Pelechano[1], Wu Wei[2,3], Lars M Steinmetz[1,2,3]

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We have developed an optimized protocol (5PSeq) including unique molecular identifiers that allows measuring all 5'Phosphate mRNA degradation intermediates present in a sample. mRNA degradation intermediates have been previously analysed to identify RNA endonucleolitic cleavage sites for 5'-3' RNA degradation mutants both in plants and yeast. However, what are the specific factors contributing to the presence of 5'P sites and how do their abundance vary across the genome and physiological conditions has not been studied.

We have investigated the 5' positions of degradation mRNAs with 5PSeq in multiple physiological conditions and drug treatments in *S. cerevisiae*. Our genome-wide analysis shows that ribosomes act as a general barrier for mRNA degradation, suggesting that the previously proposed mRNA co-translation degradation is not only possible, but also pervasive in the genome. Analysis in other organism shows that this process is evolutionary conserved. By comparing 5PSeq with Ribosome Profiling, we show that the detailed study of the mRNA degradation intermediates produced by the endogenous RNA degradation machinery allows reconstructing ribosome dynamics *in vivo*. 5PSeq is a straightforward method that does not require any polyribosome fractionation or RNAse foot-printing and that can be easily applied to any previously purified RNA. Additionally this drug-free approach can be used to study ribosome pausing sites that in alternative methods such as Ribosomal Profiling are masked by the secondary effect of drugs like cycloheximide.

We believe that 5PSeq offers a new and complementary window to study global ribosome dynamics in conditions where the use of methods requiring the use of translation inhibition drugs or the isolation of polyribosomal fractions is not possible or advisable.

Sample to Insight - analysis, visualization and interpretation of next generation sequencing and gene expression data

Anne Arens

QIAGEN GmbH, Germany; anne.arens@qiagen.com

QIAGEN provides complete sample to insight solutions for your next-generation sequencing (NGS) workflow including bioinformatics analysis. High-quality, target-enriched samples and streamlined library preparation procedures can be combined with easy-to-use, graphical user-interface-guided data analysis tools. Our solutions deliver focused and accurate results and allow you to overcome the bottlenecks in your NGS data analysis and interpretation by using our manually curated knowledgebase.

Accepting data from any major sequencing platform, CLC Genomics Workbench and CLC Cancer Research Workbench align sequencing reads to the reference genome, and call germline or somatic variants for targeted, whole exome, transcriptome and genome sequencing. Due to seamless integration of our software solutions, variants can be uploaded directly from CLC Cancer Research Workbench to Ingenuity Variant Analysis for fast and accurate filtering, annotation, and interpretation. Ingenuity Variant Analysis combines analytical tools and integrated content to help you rapidly identify and prioritize variants by drilling down to a small, targeted subset of compelling variants based both upon published biological evidence and your own knowledge of disease biology.

For gene expression analysis, CLC Genomics Workbench and CLC Cancer Research Workbench provide tools for RNA-Seq as well as expression chip support. Streamlined integration with Ingenuity Pathway Analysis allows for direct upload, analysis, and interpretation of your expression data. Ingenuity Pathway Analysis provides a rapid assessment of the signaling and metabolic pathways, biological processes, and upstream regulators that are most significantly perturbed in a dataset of interest. Ingenuity Pathway Analysis can also be used for expression data generated with other experimental platforms, including qPCR or microRNA.

Ingenuity Variant Analysis and Ingenuity Pathway Analysis leverage our unique repository of known human genes (as well as mouse and rat genes), proteins, phenotypes, variants, pathways, pathway regulators, drugs, and other compounds as well as their interactions. The Ingenuity knowledgebase is mostly composed of several millions of up-to-date experimental findings extracted from peer-reviewed journals that have been curated by MD/PhD level scientists, and third party information from public high quality databases. Recently our variant related records have been augmented by the variants reported in BIOBASE's HGMD professional database.

We will illustrate the capability of our software solutions by introducing use cases related to DNA-Seq and RNA-Seq.

High resolution NGS-based HLA-typing using in-solution targeted enrichment

Michael Wittig[1], Jarl Andreas Anmarkrud[2,3,4], Jan Christian Kässens[5], Simon Koch[6], Michael Forster[1], Eva Ellinghaus[1], Johannes E.R. Hov[2,4,7], Sascha Sauer[8], Manfred Schimmler[5], Malte Ziemann[9], Siegfried Görg[9], Frank Jacob[6], Tom Hemming Karlsen[2,4,7], Andre Franke[1]

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The human leukocyte antigen (HLA) complex contains the most polymorphic genes in the human genome. The classical HLA class I and II genes define the specificity of adaptive immune responses. Genetic variation at the HLA genes is associated with susceptibility to autoimmune and infectious diseases and plays a major role in organ transplantation and immunology. Currently, the HLA genes are characterized using Sanger- or next-generation sequencing (NGS) of a limited amplicon repertoire or labeled oligonucleotides for allele-specific sequences. We developed a highly automated HLA typing method for NGS. The method employs in-solution targeted capturing of the classical class I (*HLA-A, HLA-B, HLA-C*) and class II HLA genes (*HLA-DRB1, HLADQA1, HLA-DQB1, HLA-DPA1, HLA-DPB1*) followed by an amplification of the captured DNAs. The method was tested on 357 commercially available DNA samples with known HLA alleles obtained by classical typing. Our results showed on average an accurate allele call rate of 0.99 in a fully automated manner, identifying also errors in the reference data. Finally, the method provides the flexibility to add further enrichment target regions.

Digital PCR

Tuesday, 24/Mar/2015:
8:30am - 10:30am
Session Chair:
Jim Francis Huggett
Tim Forshew

Location:
Lecture hall 14

Presentations

How could digital PCR benefit clinical analysis?

Jim Francis Huggett
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The potential application of digital PCR to clinical analysis

Digital PCR (dPCR) is achieved by separating a PCR reaction into a large number of partitions so that a proportion contain no template. Unlike qPCR, dPCR does not require a calibration curve for quantitative analysis, offers more precise quantification as well as sensitive detection of minority genetic targets. Evidence suggests dPCR may also be more reproducible than other molecular methods, which would make it ideal for clinical applications like diagnostics. This talk will discuss the work of two European Metrology Research Programme funded projects, Infect-Met and Bio SITrace, which have investigated dPCR and highlight some of the prospective advantages as well the disadvantages. A prediction of how this method may impact on the future the application of molecular methods to clinical analysis will also be presented.

Rapid and Ultra-Sensitive Single-Cell Transcript Profiling with Droplet Digital PCR (ddPCR™): Application to neural induction

Svilen Tzonev
Digital Biology Center, Bio-Rad, United States of America; Svilen_Tzonev@bio-rad.com

Single-cell transcript profiling is undoubtedly the ideal approach for gene expression analysis in heterogenous cell types. However, the robust detection of transcripts in isolated single cells is technically challenging, especially without pre-amplification. Droplet Digital PCR (ddPCR) developed at Bio-Rad's Digital Biology Center directly counts individual molecules with superior precision and reproducibility. The ddPCR-based single-cell gene expression protocol measures even very low abundance transcripts with minimal sample processing for defined targets. Furthermore, ddPCR is performed in 96-well plates and is well suited to high throughput studies of focused sets of genes in large numbers of single cells.

In this work, we demonstrate the single-cell gene expression analysis of pluripotent P19 cells before and after neural induction. We present a simple and robust workflow for profiling multiplexed, transcript targets in flow-sorted, single cells. We characterize a panel of validated assays targeting stem cell, proliferation and differentiation marker genes including Sox2, Ki67, and EphrinB1, respectively. We compare expression levels of these genes in Retinoic Acid treated and not treated single cells and bulk RNA preparation from the same cell populations prior to sorting. We demonstrate that ddPCR provides absolute counts of transcripts from several thousand copies to less than ten copies per cell. Our findings are discussed with current data in the literature.

NGS and dPCR Analysis of Circulating Tumour DNA and Their Potential Uses in Cancer Patient Care

Tim Forshew
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Cancer is a disease of the (epi)genome. Advances in sequencing technologies have allowed rapid discovery of the genetic and epigenetic drivers of cancer. There is now growing evidence that for many solid tumours it is possible to detect these same (epi)genetic changes through the analysis of a patient's blood. This circulating and cell free tumour DNA (ctDNA) has the potential to be used as a highly specific cancer biomarker. There are a broad range of applications that are currently being intensively explored for ctDNA including non-invasive cancer detection, molecular stratification and disease monitoring.

The main challenges with the analysis of ctDNA are its dilute and fragmented nature and the often low tumour DNA fractions. Technological advances in the fields of digital PCR and next generation sequencing have recently made the detailed analysis of this DNA possible.

Using a combination of these methods we have interrogated ctDNA levels in a range of cancer types including sarcoma, ovarian cancer and breast cancer. I will outline recent results and highlight strengths and weaknesses of different analysis methods.

Droplet generation for NGS library preparation and for digital PCR on the same instrument

Sabit Delic, Manja Meggendorfer, Niroshan Nadarajah, Wolfgang Kern, Torsten Haferlach
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Objectives: The number of molecular markers used to characterize myeloid malignancies continues to constantly increase. As such, physicians and laboratories face a great unmet need to test panels of genes at a high level of sensitivity and throughput. In addition, monitoring response to therapy by analyzing mutation load of initially identified molecular markers requires extremely sensitive detection methods.

Methodology: We tested the Source instrument (RainDance, Billerica, MA) for droplet generation with subsequent next generation sequencing (NGS) or digital PCR (dPCR). For NGS we used the ThunderBolts Myeloid Panel (RainDance) composed of 53 genes with 533 amplicons. Targets of interest comprised either complete coding gene regions or hotspots. Sequencing data was generated using the MiSeq instrument (Illumina, San Diego, CA) loading 8 patients per run. 37 patients were analyzed. Results were compared to sequencing data obtained with droplet-based library preparation using the ThunderStorm (RainDance) or Access Array (Fluidigm, South San Francisco, CA) techniques.

For dPCR we used the RainDrop System (RainDance). We established 3 assays for the genes *RUNX1, NPM1* and *ASXL1* and analyzed 83 patients resulting in 160 samples in total. With the *RUNX1* assay (mutation c.521G>A) 35 patients were analyzed. The*NPM1* assay (c.863_864insCTTG) was used to analyze 19 patients and 28 patients were analyzed with the *ASXL1* assay (c.1934dupG). Droplet generation was performed with genomic DNA for *RUNX1* and *ASXL1* assays and cDNA for the *NPM1* assay. The results were compared to known mutation loads as detected formerly by NGS (*RUNX1*), dPCR using the Fluidigm EP1 system (*NPM1*) or Sanger sequencing (*ASXL1*). In 76/160 samples these methods had proven the absence of the respective mutations or the presence at minimal residual disease (MRD) levels <10%. Over all samples were a mutation was detectable the mutation load ranged from 0.1% to 93%.

Results: We could show that droplet generation using the Source instrument can be applied in clinical testing. NGS libraries generated by the Source instrument and analyzed on MiSeq instruments gave basically the same results as obtained by our well established routine methods. All

known mutations have been identified with a comparable mutation load and good coverage. The dPCR experiments performed on the RainDrop system gave results correlating very well with the known mutation loads as analyzed by NGS, EP1 or Sanger sequencing, respectively.

Conclusion: We here demonstrated that droplet-based sample preparation enabled to target 53 candidate genes for next generation sequencing in myeloid malignancies in a routine diagnostic environment. In addition, we showed that using the same instrument the droplet-based sample preparation can also be applied to dPCR for monitoring MRD. Thus the Source instrument provides a feasible 2-application droplet generation platform for both, NGS library preparation and dPCR in clinical testing.

Non-coding RNAs 1

Tuesday, 24/Mar/2015:
11:00am - 12:30pm
Session Chair:
Jo Vandesompele
Robert Sjöback

Location:
Lecture hall 14

Presentations

Updated evaluation of quantitative miRNA expression platforms in the microRNA quality control (miRQC) study

Pieter Mestdagh, Jo Vandesompele
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MicroRNAs are important negative regulators of protein-coding gene expression and have been studied intensively over the past years. Several measurement platforms have been developed to determine relative miRNA abundance in biological samples using different technologies such as small RNA sequencing, reverse transcription–quantitative PCR (RT-qPCR) and (microarray) hybridization. In this study, we systematically compared 14 commercially available platforms for analysis of microRNA expression. We measured an identical set of 20 standardized positive and negative control samples, including human universal reference RNA, human brain RNA and titrations thereof, human serum samples and synthetic spikes from microRNA family members with varying homology. We developed robust quality metrics to objectively assess platform performance in terms of reproducibility, sensitivity, accuracy, specificity and concordance of differential expression. The results indicate that each method has its strengths and weaknesses, which help to guide informed selection of a quantitative microRNA gene expression platform for particular study goals.

MicroRNA profiling using a rapid, highly sensitive qPCR panel

Simon Baker[1], Lihan Zhou[2], Florent Chang Pi Hin[1], Ruiyang Zou[2], Heng-Phon Too[2]
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Recent studies have shown that microRNAs are released from tissues and cells into circulation where their expression correlates with specific disease conditions. These microRNAs are protein bound or encapsulated in vesicles and remain stable in biofluids. Such attributes made circulating microRNAs promising non-invasive candidate biomarkers for the early detection of disease, prognosis or treatment selection. However, significant technological challenges remain. These include data inconsistencies across studies which has hampered the development of accurate and robust miRNA-based tests. Efficient identification of miRNA biomarkers requires a well-designed study and an integrated workflow with consistent sample extraction, robust RT-qPCR quantification, and stringent statistical analysis. In addition, control measures are essential to monitor and normalize technical or biological variations that may obstruct the interpretation of data. We have developed an enabling platform based on a proprietary design concept and validated the resulting qPCR assays against both clinical samples and synthetic RNA constructs. We have detected extremely low levels of circulating microRNAs with high sensitivity and specificity. This new platform has been used for the routine detection of over 400 microRNAs, using low input material (e.g. 200 microliters of serum and plasma) with a dynamic range span over eight orders of magnitude. The total workflow variation was less than 0.5 Ct. The platform has been validated independently by molecular diagnostic and pharmaceutical laboratories and showed superior performance to existing commercial assays. This highly reproducible, sensitive platform has been developed to produce complete systems for miRNA profiling of cancer samples, biofluids and stem cells.

Fast and flexible bioinformatics analysis of RNA seq data to provide biological insight

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In recent years, Next Generation Sequencing (NGS) has evolved from a novel technology to become an established method for investigating biological systems at the genetic level. Furthermore, ongoing advances in the underlying chemistry and associated instrumentation have led to the development of a number of semi-automated platforms that can be applied to a range of biological problems. In this way biological samples may be sequenced in a semi-automated and relatively trouble free manner.

However, analysis of the generated data remains a major obstacle; even a small sequencing run can generate vast quantities of data. While there are many commercial and open source software solutions available for analyzing NGS data, running these tools effectively in an automated manner remains a formidable challenge. Also, while an automated analysis can provide a general overview of the data (for example, mapping differentially expressed features, and Gene Ontology and Pathway enrichment) it does not generally provide deeper insight into the system under investigation. Furthermore, even for this basic analysis, the quantity of data generated can be overwhelming. Thus, more in-depth investigation and interpretation of raw sequence data remains an elusive task for many researchers who lack access to bioinformatics expertise and computing resources.

We have developed a flexible “plug and play” bioinformatics platform that allows customizable NGS analyses which can be tailored to specific needs on a case by case basis for RNA sequencing. The platform has the flexibility to incorporate existing steps or permit the creation of new steps which perform novel analyses in terms of method, reference data, or data visualization. In this way, a NGS dataset can be mined in greater depth to yield deeper insight into an experiment or biological system. For microRNA sequencing, our unique XPloreRNA™ App is integrated into the data analysis to facilitate gene ontology mediated assessment of biological significance. This NGS platform complements our existing bioinformatics and experimental services business, built on extensive experience gathered from the profiling of almost 30.000 clinical samples on our microarray and qPCR platforms within a highly controlled laboratory infrastructure.

In this presentation we will provide an overview of the bioinformatics platform and present some specific examples of its application to real NGS datasets as well as results from quality control studies using test data.

Life Technologies: Lunch-time seminars

Tuesday, 24/Mar/2015:
1:00pm - 2:00pm

Location:
Lecture hall 14

Presentations

Digital PCR analysis of tumor-specific mutations from peripheral blood in ER positive breast cancer samples

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Background**:** Digital polymerase chain reaction (dPCR) is a new technology that enables detection and quantification of tumor-specific mutations in circulating plasma DNA. Detecting tumor-specific mutations in circulating plasma DNA may potentially be useful to select systemic therapies for solid tumors as well as to monitor treatment outcome. The aim of our study is to evaluate the feasibility of QuantStudio® 3D Digital PCR System for detecting and quantifying PIK3CA mutations from plasma of breast cancer patients.

Methods**:** 49 Formalin-fixed paraffin-embedded (FFPE) stage IV tumor samples were screened for PIK3CA mutations using cobas® 4800 System (Roche Molecular Diagnostics). Plasma samples were analyzed by digital PCR using Rare Mutation Assays for E542K, E545K and H1047R on QuantStudio® 3D Digital PCR System (Life Technologies). We compared the tumor markers CA 15-3 and CEA with the PIK3CA mutation quantification in circulating free DNA (cfDNA) from plasma.

Results**:** Of the 49 ER positive breast cancer tumors analyzed, 20 harbored a mutation in PIK3CA. Regarding tumor mutation status, the proportion of observed agreement between COBAS and digital PCR was 100%. However, a moderate agreement was found between FFPE samples and ctDNA, which might be due to the heterogeneity of metastasic disease. Circulating tumor DNA was successfully detected in 12 of plasma samples. The sensitivity of digital PCR assay allowed for the detection of a mutant allele fraction of lower than 1% (detection level guaranteed by Life Technologies). In 9 women with PIK3CA somatic mutation, circulating tumor DNA and tumor markers CA15-3 and CEA were quantified in a total 29 serial plasma samples. Overall, the level of PIK3CA mutations correlated with the amount of tumors markers.

Conclusions**:** The methodology presented in this study is a feasible approach for PIK3CA mutation detection and quantification in blood derived samples.

Highly sensitive amplicon-based RNA quantification by Ion Torrent Proton semiconductor sequencing.

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The phenotype of a living cell is determined by its pattern of active signaling networks. Thus, a subset of the transcriptome can be used to define the “molecular phenotype” based on gene expression analysis. Digital amplicon based RNA quantification by deep sequencing allows multiparallel gene expression analysis for molecular phenotypic screens as a novel tool to monitor the state of biological systems. We show that the activity of signaling networks can be assessed based on a set of established key regulators and expression targets rather than the entire transcriptome. We compiled a panel of 917 human *pathway reporter genes*, representing 154 human signaling and metabolic networks for integrated knowledge- and data-driven understanding of biological processes. We show several examples in which reporter genes deliver an accurate pathway-centric view of the biological system under study. Finally, we show for the first time application of AmpliSeq-RNA for analysis of gene expression of a single cell.

Biomarker Discovery & Circulating Nucleic Acids

Tuesday, 24/Mar/2015:
2:00pm - 6:30pm
Session Chair:
Stephen Andrew Bustin & Michael W. Pfaffl

Location:
Lecture hall 14

Presentations

Mass Spectrometry Based Draft Of The Human Proteome

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Proteomes are characterized by large protein-abundance differences, cell-type- and time-dependent expression patterns and post-translational modifications, all of which carry biological information that is not accessible by genomics or transcriptomics. Here we present a mass-spectrometry-based draft of the human proteome and a public, high-performance, in-memory database for real-time analysis of terabytes of big data, called ProteomicsDB. The information assembled from human tissues, cell lines and body fluids enabled estimation of the size of the protein-coding genome, and identified organ-specific proteins and a large number of translated lincRNAs (long intergenic non-coding RNAs). Analysis of messenger RNA and protein-expression profiles of human tissues revealed conserved control of protein abundance, and integration of drug-sensitivity data enabled the identification of proteins predicting resistance or sensitivity. The proteome profiles also hold considerable promise for analysing the composition and stoichiometry of protein complexes. ProteomicsDB thus enables navigation of proteomes, provides biological insight and fosters the development of proteomic technology.

CircRNA: a new class of non-coding ribonucleic acid molecules

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Although the existence of circular RNAs (circRNAs) has been known for over a decade, it was not until recently that the scientific community became aware of how widespread this phenomenon is. In fact recent reports, based on high-throughput sequencing of ribosome-depleted RNA, suggest that hundreds of circRNAs are expressed in mammalian cells. It has also been reported that due to their structure circRNAs show increased stability compared to linear RNA, which makes them interesting candidates for biomarker studies.

This talk will give an overview on the current state of knowledge about circRNAs, their detection in RNA-seq data and methods of experimental validation of specific candidates. In addition preliminary findings on circRNA-expression in the heart will be presented.

Rapid High Sensitivity Monitoring of Tumor Genetic Changes for Targeted Therapy of Cancer

Michael Joseph Powell, Aiguo Zhang, Madhuri Ganta, Elena Peletskaya

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Genetic variations in human cancer genes can determine whether a patient will respond to a specific therapeutic agent. For example patients with Non-Small Cell Lung Cancer (NSCLC) whose tumors carry mutations in the Epidermal Growth Factor Receptor (EGFR) can be responsive to tyrosine kinase inhibitors (TKI's) or their tumors can develop resistance to these therapeutics agents. Colorectal cancer patients that carry a single nucleotide change in Exon 2 of the KRAS proto-oncogene are unresponsive to ant-EGFR antibody therapy. Thus there is a need for a rapid, sensitive way to detect mutations in patient tumor derived DNA especially a minimally invasive technique that does not require surgery. QClamp is a real-time PCR method that can detect mutations in tumor derived DNA within a few hours from patient sample procurement. The technology allows the precise and sensitive detection of genetic variations without the need for any novel instrumentation.

Proximity Ligation Assay for the specific detection of *Aspergillus* species

Gemma Johnson[1], Mark Shannon[2], Christopher Thornton[3], Samir Agrawal[4], Cornelia Lass-Flörl[5], Wolfgang Mutschlechner[5], Stephen Bustin[1]

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Antibody- and real-time quantitative (qPCR)-based assays are proving useful for a more sensitive and specific identification of*Aspergillus* in clinical samples. However, both approaches have important shortcomings: the relative insensitivity of current antibody-based strategies makes them less suitable for earliest possible diagnosis, whereas the mere detection of pathogen DNA is uninformative with regards to its viability or infectivity. Hence the ultimate assay would combine the specificity of pathogen-specific antibody detection with the sensitivity of qPCR. The proximity ligation assay (PLA) uses qPCR to detect the interaction of antibodies with their specific antigens.

We have used this technology with an *Aspergillus* specific monoclonal antibody (MAb) to demonstrate sensitive and specific detection of*Aspergillus* mannoprotein in clinically relevant samples. The assay is simple to use, rapid and significantly more sensitive than other antibody-based assays in current use. This has important implications for early diagnosis and targeted treatment of Invasive Aspergillosis.

Extracellular Vesicles As Disease Biomarkers?

Mercedes Tkach[1,2], Joanna Kowal[1,2], Clotilde Thery[1,2]

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Eukaryotic cells release different types of membrane vesicles into their extracellular environment that mediates communication with the surrounding cells. Among them, exosomes are small (30-100 nm) vesicles formed by inward budding inside the lumen of endosomes, which then fuse with the plasma membrane to release extracellularly their internal vesicles. Once released, exosomes can either bind to surface receptors and/or be internalized, or directly fuse with a target cell. Their cargo, including specific proteins, lipids and nucleic acids, can induce various forms of intracellular signalling, thus modifying the physiological state of the recipient cell in a very complex way.

The function of exosome secretion in the context of tumor development has been extensively studied in the past years. On the one hand, exosomes secreted by tumor cells can be captured by dendritic cells and induce anti-tumor immune responses. On the contrary, it has been shown that they can also actively suppress tumor-specific immune responses. Moreover, it has also been demonstrated that tumor derived exosomes can transfer angiogenic proteins or oncogenes from one cell to another, thus promoting tumorigenesis and metastasis.

Exosomes were found in several body fluids. In particular, numerous studies show that cancer patients have higher concentrations of circulating exosomes, a feature that correlates with disease progression. As these exosomes can carry tumor molecules, there has been an increasing interest in the study of circulating exosomes as a source of biomarkers in cancer patients. This was further sparked upon the discovery of mRNA and miRNA in exosomes. These nucleic acids are specifically loaded into the lumen of exosomes and can be then transferred to other cells regulating their gene expression. Several groups have detected an altered RNA cargo in body fluid-derived exosomes from patients with different diseases, strongly encouraging the use of exosomes as biomarkers for diagnosis and prognosis.

However, we want to stress that the actual nature of the vesicles analyzed is not always clearly demonstrated. Depending on the specific intracellular site of vesicle formation, their constitutive components differ from one type of vesicles to another and so the functions and the utility as biomarkers may be different. We have already shown that the vesicles obtained with the classical differential ultracentrifugation method are heterogeneous in their size, density, composition and biogenesis. More recently, we analyzed these vesicles in detail, using novel strategies to separate them and performed a side-by-side comparison of their protein composition. This allowed us to identify novel markers specific for the different vesicles secreted by a cell. In our opinion, this type of analysis should be done in different cell types in order to determine if all, or some, or none of these vesicles could be relevant as disease biomarkers.

Cell to Cell Communication via extracellular RNA and Exosomes.

Michael W Pfaffl, Benedikt Kirchner, Dominik Buschmann, Melanie Spornraft

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Exosomes are cell-derived vesicles that are present in many tissues and in all biological fluids, including blood, milk, urine, and sweat. Exosomes are proposed to have specialized functions and play a key role in cell-to-cell communication, e.g. in the immune system, in cancer progression, in intercellular signalling, and in cellular waste management. Consequently, there is a growing interest in molecular diagnostic and the clinical application. Very less information is available about the exosomes and their small RNA composition in biofluids such as milk and whether milk possesses its own defined small RNA profile.

First, an optimized RNA isolation method combined with small-RNA Seq was established to profile microRNAs (miRNAs) and a very emerging new class of small RNAs the piwi-interacting RNAs (piRNAs), in bovine plasma and whole blood. The presented data evaluation pipeline offered to analyze data regarding quality and allowed annotation and generation of readcount tables by successive reduction of complexity of data sets and aligning sequencing reads to reference databases.

Second, exosome isolation methods were established for high-yield and high-quality isolation from blood and milk. Exosomes were isolated by differential ultracentrifugation and density gradient centrifugation and further analyzed by western blotting for CD63 and MFGE8 as a general and milk-specific marker, respectively. To assess extracellular vesicles populations, Nanoparticle Tracking Analysis was used for the evaluation of particle count and diameter in distinct gradient fractions. Additionally, protein quantification (BCA assay) and RNA analysis (RT-qPCR) were also performed in exosome-containing fractions.

To generate a holistic overview of all present small RNAs Next Generation Sequencing (small RNA-Seq) was performed on whole blood, plasma, whole milk, and exosomes. Small RNA-Seq was performed using an Illumina HiSeq 2500 and subsequent data analysis was done independently, using either the GGS and GGA stations from Genomatix Software or using freely available python scripts and R-packages. To

validate these findings key microRNAs were quantified via RT-qPCR for fold change comparisons. Experimentally validated mRNA targets for regulated microRNAs were taken from the Tarbase 6.0 database from Diana Lab and pathway analyses were generated using Genomatix Pathway System.

RNA Seq clearly showed that milk and exosomes possesses its own unique small RNA profile compared to blood. It highlights its dynamic changes during the first lactation days. Pathway analysis for affected targets revealed significant impact on cell cycle progression, cell adhesion, DNA repair, apoptosis, and oncogenic defense. This study underlines the potential role of microRNAs (and small RNAs in general) in mammary gland physiology and cell-to-cell communication. Milk microRNAs and exosomes seem essential for the mammary gland immune system, but also as an active modulator in the newborn calf.

Exosomes – A novel approach to biomarker identification

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The promise of circulating biomarkers is to enable minimally-invasive monitoring of specific molecules that yield predictive or prognostic information. Liquid biopsies enable us to monitor the evolution of genetic aberrations in primary tumors as they shed exosomes into the circulation. The major limitation of this strategy is the ability to isolate these exosomes in a standardized manner. QIAGEN has developed solutions to help isolate and enrich exosomes and other extracellular vesicles, to allow the detection of low-abundance RNAs that are in circulation, or to profile miRNAs that establish a specific expression signature. The seminar will describe solutions from sample preparation to molecule detection for screening, profiling, and monitoring putative biomarkers. The application examples will highlight new tools for exosomes, and circulating miRNA.

RT-qPCR based screening of blood-circulating microRNAs for the identification of biomarkers and novel drug targets

Matthias Hackl[1], Sylvia Weilner[1], Susanna Skalicky[1], Rita Reynoso[2], Natalia Laufer[3], Heribert Stoiber[4], Peter Dovjak[5], Peter Pietschmann[6], Johannes Grillari[1,2]
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Introduction: MicroRNAs (miRNAs) are small non-coding RNAs that regulate gene expression and are known to take part in the control of various biological processes. Recently, miRNAs have been identified to be secreted into the bloodstream from cells of various tissues, thus, possibly indicating local pathological processes. Therefore, there is a growing interest in circulating miRNAs as minimal-invasive diagnostic targets or as potential targets for drug development.

Methods: We have implemented a commercial LNA-based RT-qPCR method for screening of hundreds of miRNAs in cell-free blood samples. Pre-analytical variability in terms of sample storage, type, and freeze/thawing was assessed, and analytical variability was controlled using a combination of 5 spike-in controls added at various stages during the analysis workflow. Based on pilot studies, a discovery panel for circulating miRNAs analysis with 375 primer pairs was designed and used in the course of two research studies.

Results: The first case study was designed to identify changes in circulating miRNAs in response to recent osteoporotic fractures at the hip, a major health burden in aging populations of developed countries. In an exploratory analysis of 7 fracture and 7 control patients, six miRNAs with significant (adj. $p < 0.05$) changes in serum levels were identified. Clustering and ROC analyses showed that these miRNA together provided excellent discrimination of case and control samples. Subsequent validation in an independent cohort of 23 patients (11 control, 12 fracture) confirmed significant regulation for three miRNAs. These miRNAs were selected for in vitro testing using an in-house developed bioassay for bone formation, in order to evaluate correlative or causative relation to fracture-risk.

The second case study aimed to identify circulating miRNAs specific to elite HIV controllers. These patients are known to maintain HIV-1 viral loads below the limit of detection, albeit being infected with HIV-1. For that purpose EDTA-Plasma from HIV elite controllers (n=9) and chronically infected individuals (n=9) was compared against healthy donors (n=6). Unsupervised cluster analysis and statistical comparison identified strong difference in circulating miRNA patterns between chronic HIV infected patients and healthy controls, while no differential expression between elite patients and healthy controls could be observed. Three miRNAs, hsa-miR-29b-3p, hsa-miR-33a-5p and hsa-miR-146a-5p were found to be significantly higher in plasma from elite controllers than chronic infected and therefore selected for in vitro testing. The results clearly show that hsa-miR-29b-3p and hsa-miR-33a-5p overexpression reduced the viral production in MT2 and primary T CD4+ cells.

Conclusion: These data clearly show that the analysis of circulating miRNAs using a robust RT-qPCR pipeline is not only suitable for biomarker identification but also a viable approach for drug target identification.

Next Generation Sequencing 2

Tuesday, 24/Mar/2015:
8:30am - 10:30am
Session Chair:
Karen E. Nelson & Michael W. Pfaffl

Location:
Lecture hall 15

Presentations

Next Generation Sequencing Applications to the study of human and animal Microbiomes

Karen E. Nelson
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Advances in sequencing technologies and analytic approaches have enabled an in-depth characterization of the microbial communities that are associated with animals and humans, and have increased the possibilities for improving our understanding of human and animal health and disease. In humans, we now recognize that these microorganisms are essential for healthy growth and development. Disruptions of our normal microbial populations have been implicated in several diseases including colon cancer, autism, acne, and various gastrointestinal disorders to name a few. The opportunities presented through studying the microbiome are enormous and range from the development of new probiotics, novel diagnostics for infectious diseases, and treatments that include using the microbiome as a therapeutic. Similar studies are now being performed on a variety of animal species, including companion animals, those used for research, agricultural and food purposes. Animal microbiome studies are allowing us to broaden our understanding of their microbiomes and related applications, including animal feed

conversion efficiency, microbes at the animal-human interface, and implications for infectious disease control and prevention strategies. In addition, various animal models allow for the design of controlled microbiome experiments to apply to human-microbiome interactions in health and disease.

DNA-based Food Authenticity and Safety Control using Advanced Barcoding by Next Generation Sequencing

Ilka Haase, Christine Käppel, Elmar Schilling
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DNA-based methods have become a common tool for the analysis of plant, animal and also microbial species in food and feed. But especially since the horse meat scandal in 2013, authenticity as well as safety control of food and feed by DNA is on the spotlight. Sanger Sequencing of several barcoding regions is the state of the art analysis for animal, plant and microbial species identification in food and environmental samples with unknown composition. However, the identification and determination of species in complex products by this approach is challenging due to the widespread diversity of species that might be present and the overlay of the sequences of all species in the Sanger chromatogram. This tool is therefore perfectly suited for to the analysis of pure samples but is limited to mixtures of at most two species. Eurofins is therefore developing Next Generation Sequencing (NGS) methods for the species identification in mixed samples to overcome this limitation. Many NGS publications concerning metagenomics/metabarcoding studies focus predominantly on microbial populations in environmental samples. In the meantime, also zoological studies use NGS for the analysis of faecal samples of herbivores as well as carnivores in order to get insights into their diets and habitats. In the food and feed sector, NGS is so far only mainly used for the analysis of food-borne pathogens and starter cultures in fermentation processes. However, constantly declining costs in the NGS field makes the methods attractive also for routine analysis of food, feed and environmental samples in the future. The oral presentation will give an overview on the ongoing developments at Eurofins.

Integrative Analyses of Primary and Relapse Tumor Samples Identifies Genomic Changes During Cancer Progression

Alexander Schramm
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The rise of next-generation sequencing approaches to comprehensively assess and interpret the molecular genetic tumor landscape has fueled efforts to tailor medical care for individual cancer patients. While progress in therapies has been significant for many cancer types, death of cancer results, in the vast majority of cases, not from the initial disease manifestation, but from metastasis or recurrence. This helds also true for neuroblastoma, the most common extracranial tumor of childhood. In this particular tumor, therapies are quite successful in reducing the initial tumor burden, but relapses cannot be treated with curative intent. In order to identify specific genetic aberrations at recurrence of disease, we performed whole-exome sequencing of neuroblastoma at relapse and compared their mutational profiles to the corresponding primary tumors and constitutional DNAs. These data were integrated with arrayCGH, methylation and mRNA expression profiles and validated using preclinical NB models. Global allele frequency analyses suggested an enrichment of pre-existing mutations during disease progression. None of the patients presented with acquired mutations in previously identified NB driver genes at relapse. Exome sequencing and phylogenetic reconstruction using multiple relapses from a single patient in our cohort provided unique insight into molecular genetic evolution in neuroblastoma. Clonal branches evolved from the primary tumor gave rise to independent relapses colonizing different sites. To assess not only spatial but temporal tumor evolution, we also analyzed allelic frequencies of mutated genes deduced from both exome sequencing and amplicon resequencing in primary-relapse tumor pairs in our patient cohort. Results from both techniques suggest that most relapse-specific mutations evolve either de novo or from a rare subpopulation present in the primary tumor. From a global view, clonal selection during disease progression and relapse can occur independently at various time points and metastatic localizations. These findings underpin the need for multiregional sequencing when more than one relapse tumor is detected in a single patient in order to define promising individualized therapy approaches.

Microbiomes via sequencing: Challenges and opportunities

Thomas Clavel
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The last decades have witnessed the advent of molecular techniques for the study of complex microbial communities. In particular, high-throughput sequencing has become very popular for rapid and in-depth assessment of microbiomes in targeted or shotgun manners. In as much as these approaches delivered novel insights into many different ecosystems and open ways to so far unexplored diversity, limitations have been often neglected, especially in rapidly expending areas of research such as those around the mammalian gut microbiome. In the present talk, I will address issues about standardization and the need to go beyond single studies for global assessment of bacterial communities using 16S rRNA gene sequencing.

NGS data analysis

Tuesday, 24/Mar/2015:
11:00am - 12:30pm
Session Chair:
Robert P. Loewe & Michael W. Pfaffl

Location:
Lecture hall 15

Presentations

Benchmarking of RNA-seq data processing pipelines using whole transcriptome qPCR expression data

Jan Hellemans[1], Jo Vandesompele[1,2], Pieter Mestdagh[1,2]
[1]Biogazelle, Belgium; [2]CMGG, UGent, Belgium; jan.hellemans@biogazelle.com

RNA sequencing is becoming increasingly popular to perform transcriptome wide gene expression analyses. The recently published SEQC study assessed the performance and key characteristics of RNA-seq by sequencing the MAQC samples to very deep coverage. We have extended this study by detailed comparison of the results generated by different data processing pipelines against those obtained by transcriptome wide qPCR measurements. The relative performance and differences between 4 pipelines (Sailfish, tophat-HTseq, star-HTseq and tophat-cufflinks) as well as their concordance to qPCR data will be presented.

Chances and challenges in RNA-Seq analysis

Matthias Scherf, Martin Seifert
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RNAseq is a next generation sequencing based technology that facilitates gene expression detection at a high resolution level. It allows to measure and (semi) quantify RNA levels as well as differential analysis. In addition, structural detection of transcripts and splice variants becomes feasible. This is especially helpful for "non-mainstream genomes" as it enables de-novo transcript assemblies and annotation.

Besides these chances also significant challenges have to be overcome. The most important aspect is the inherent diversity and complexity of RNA species and the multitude of potential transcript and splice variants. In this respect, today's NGS technologies are mainly limited by the read length. Read length also plays a role for mapping positions with insufficient uniqueness. For RNA analyses the correct measurement of paralogous genes are one example. To address these issues different aspects have to be taken into account:

1. The background transcriptome annotation should be as complete as possible
2. Mapping strategies should take the uniqueness into account
3. Visualization is an integral step for consistency evaluation

RNAseq data analysis: getting more insight by combining it with public RNAseq experiments

Philip Zimmermann
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Most biological experiments are analyzed individually by comparing experimental factors being tested in that particular experiment. Typically, in microarray or RNAseq experiments, one would try to identify genes and processes having significantly altered expression levels in the tested conditions relative to control conditions. The main drawback with this approach is that it merely provides genes showing altered expression, but it doesn't indicate whether they are SPECIFIC for that factor. For example, inflammatory genes are upregulated in a wide variety of diseases and frequently appear in the list of most significantly regulated genes when comparing diseased with healthy control samples. To find which inflammation genes are specific for one chosen disease therefore requires that this particular disease is analyzed in combination with datasets from other diseases. In this talk, I will show how RNAseq or microarray experiments can be better analyzed and interpreted when analyzing them in combination with chosen public experiments or with reference datasets.

Agilent Technologies: Lunch-time seminars

Tuesday, 24/Mar/2015:
1:00pm - 2:00pm

Location:
Lecture hall 15

Presentations

High Resolution Melting – How To Produce Highly Reproducible Measurements

Robert P. Loewe
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High resolution melting (HRM) has become a prominent feature in qPCR instrument and chemistry line up. The ordeal however of designing a valid HRM experiment from the primer sequence to amplicon size and designating the proper controls for each unique question remains. Some obstacles are of cause greatly debated in literature and these will be summarized, but additionally a short guide to achieve a successful result is needed and given here. Touching the topics of primer design for sufficient amplicon length, the great focus will be experimental design and genotype calling by curve discussion. Furthermore the non-trivial situation underlining somatic mutations is debated, where frequency of single nucleotide exchanges can range from none to present in all cells. Aim is to give people an insight for this highly relevant method.

Total Confidence qPCR – The New AriaMX Real-Time PCR System

Bernd Martin
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Total Confidence qPCR – The New AriaMX Real-Time PCR System

The AriaMx is a fully integrated quantitative PCR amplification, detection, and data analysis system. The system design combines a state-of-the-art thermal cycler, an advanced optical system with an LED excitation source, and complete data analysis software. The AriaMx amplifies your productivity with its unique modular and agile design, intuitive touch-screen interface, advanced, easy-to-use reporting, and over 120 attributes monitored via the built-in on-board diagnostics to help pinpoint assay or instrument issues as they arise. The instrument can hold up to six optics modules, and the scanning optics design delivers optimal separation between the dyes and between samples. The instrument provides a closed-tube PCR detection format that can be used with a variety of fluorescence detection chemistries including SYBR® Green and EvaGreen dyes as well as fluorogenic probe systems including TaqMan probes.

Microgenomics & Single-Cell Diagnostics

Tuesday, 24/Mar/2015:
2:00pm - 6:30pm
Session Chair:
Anders Ståhlberg & Mikael Kubista

Location:
Lecture hall 15

Presentations

Expression Profiling of Circulating Tumor Cells: a Prognostic and Predictive Biomarker in Cancer.

Mikael Kubista[1,2], Robert Sjöback[1], Marie Jindrichova[2], Eva Rohlova[1], Vendula Novosadová[1,2], Siegfried Hauch[3], Katarina Kolostova[4], Bahriye Aktas[5], Mitra Tewes[5], Maren Bredemeier.[5], Sabine Kasimir-Bauer[5]
[1]TATAA Biocenter, Sweden; [2]Institute of Biotechnology, CAS, Czech Republic; [3]AdnaGen; [4]Charles University; [5]University Hospital Essen; mikael.kubista@tataa.com

We have developed a strategy to predict response to treatment of cancer patients based on expression profiling of circulating tumour cells (CTCs). CTCs are collected from blood using AdnaGen immunomagnetic capture followed by expression profiling with TATAA Biocenter GrandPerformance assays via preamplification. Preliminary results from a pilot study on breast cancer (BC) patients demonstrates excellent sensitivity, technical reproducibility and identifies a set of genes that separates a group of non-responders. The approach shows great promise as liquid biopsy for the motoring of treatments and prediction of responses.

Revolutionizing our view of the genome and transcriptome through innovative sequencing workflows

Dave Ruff, Marc Unger, Jay West, Richard Fekete, Robert Jones
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Single-cell science is opening vast new frontiers for biological discovery. Recent advances in integrated fluidic circuits (IFCs) have enabled the development of specialized technologies for integrating cell and molecular analysis at the single-cell level: the Fluidigm® C1™ system. C1 readily integrates single-cell isolation and generation of material for qPCR, miRNA, protein, and next-generation sequencing analysis of gDNA and mRNA. We now introduce two new additions to our suite of single cell technologies: the high-throughput RNA sequencing IFC and the Polaris™ system. This second-generation C1 IFC, with 800 cell-capture sites, provides higher-throughput RNA sequencing to facilitate cell characterization, cell state interrogation, and systems biology studies. Barcoding of individual cells on the IFC permits simple and efficient cell-to-sequence workflows. The Polaris system uniquely enables active cell capture and manipulation. Researchers will be able to select individual cells from a population based on multiple fluorescence markers, precisely control the environment to which the individual cells are subjected, and profile each cell for the mRNA response to these environmental perturbations and/or dosing regimens. This active and prescribed manipulation of individual cells will enable profound insights into previously unknown biological signaling and response pathways and mechanisms. Taken together, C1, the High-throughput RNA sequencing IFC, and Polaris present the scientific community with disruptive suite of tools that will revolutionize biology.

From Cultures to Results in One Day: A New Workflow for Accurate and Reproducible Large Scale qPCR Expression Analysis from Limited Samples in Stem Cell Research

Mark Kibschull[1], Stephen Lye[1,2,3], Steven Okino[4], Haya Sarras[5]
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Several applications in the stem cell field use RT-qPCR to routinely assess the quality of cell cultures by analyzing specific sets of marker genes. For instance, during lineage differentiation of human embryonic stem cells (hESC) into defined cell types, or, vice versa during reprogramming of somatic cells into induced pluripotent stem cells (iPSC), researchers need to assess the performance of their culture systems frequently. Often, only a small sample of a stem cell culture can be collected without compromising long-term experiments. Although RT-qPCR is highly sensitive, a small sample sizes may restrict the number of targets that can be analyzed, and amplification steps that are applied to increase starting material may introduce bias in gene expression quantification.

Here, we present a 4-step workflow using validated reagents, allowing the (i) direct, single-step generation of gDNA-free total RNA lysates from micro samples of hPSC colony biopsies or individual embryoid bodies. The lysate is fully compatible with (ii) reverse transcription reagents to produce cDNA. In a (iii) target-specific multiplex pre-amplification reaction, using validated primers, 100 cDNA targets are 1000 fold enriched, and thereby providing sufficient material for subsequent (iv) SYBR-Green based qPCR analysis. We show that this workflow allows fast and reproducible gene-expression profiling, and introduces only minimal bias (<0.75 Cq values) during the pre-amplification step, when data are compared to the non-amplified starting cDNA samples.

When comparing individual samples of the hESC line CA1, collected and processed at different time points, and consisting of varying sample sizes (500-50,000 cells), this workflow delivers excellent and unbiased correlations in normalized gene-expression data.

We demonstrate that during iPSC derivation, individual colonies can now be analyzed for hundreds of different markers during the reprogramming process, by using only small clusters of cells collected from culture plates. Also, during long-term embryoid body (EB) culture experiments the differentiation of specific cell types can be monitored by lysing and processing as little as individual EBs.

In conclusion, this workflow allows accurate transcriptional profiling of micro-samples for hundreds of targets using SYBR-Green-based RT-qPCR analysis within one work day. Therefore, it provides a rapid, more cost-effective method to screen and evaluate various types of stem cell cultures without compromising continuing experiments. This allows researchers not only to reduce individual culture formats, costs and processing time, but also to increase sample throughputs and generate reliable gene-expression data from limited or precious biological material.

Feasibility of a workflow for the molecular characterization of single Circulating Tumor Cells by Next Generation Sequencing.

Francesca Salvianti[1], Giada Rotunno[2], Francesca Galardi[3], Francesca De Luca[3], Marta Pestrin[3], Alessandro Maria Vannucchi[2], Angelo Di Leo[3], Mario Pazzagli[1], Pamela Pinzani[1]

[1]Department of Clinical, Experimental and Biomedical Sciences, University of Florence, Florence Italy.; [2]Department of Clinical and Experimental Medicine, University of Florence, Florence Italy; [3]Sandro Pitigliani Medical Oncology Department, Hospital of Prato, Istituto Toscano Tumori, Prato, Italy; p.pinzani@dfc.unifi.it

Circulating Tumor Cells (CTCs) represent a “liquid biopsy of the tumor” which might allow real-time monitoring of cancer biology and therapies in individual patients. CTCs are extremely rare in the blood stream and their analysis is technically challenging. The purpose of the study was to explore the feasibility of a protocol for the molecular characterization of single CTCs. CTCs were enriched and enumerated by CellSearch in blood samples collected from four metastatic breast cancer patients and subsequently isolated by DEPArray to obtain single CTCs to be submitted to Whole Genome Amplification. Samples (3-5 single CTC per patient) were analysed by NGS on the Ion Torrent system using the Ion AmpliSeq™ Cancer Hotspot Panel (Thermo Scientific) able to investigate genomic "hot spot" regions of 50 oncogenes and tumor suppressor genes. We found 53 sequence variants in 26 genes: 36 variants with possible deleterious consequences and 17 supposed benign variants on the basis of the Polyphen software. Twenty-two mutations were already reported in the COSMIC database. The gene with the highest number of sequence variants is TP53 (with 10 variants) followed by PDGFRA (5 variants) and KIT (4 variants). We observed inter- and intra-patient heterogeneity in the mutational status of CTCs. In 3 patients we could compare the NGS results from CTC with those from the primary tissue evidencing few mutations common to the two different compartments. The discordance between the mutational status of the primary tumor and CTCs suggests that CTCs in advanced stages may reflect the disease status better than the primary tumor. This study demonstrates the feasibility of a non-invasive approach based on the liquid biopsy in metastatic breast cancer patients.

Single-cell analysis of Myxoid liposarcoma reveals novel subpopulations and dysfunctional cell cycle regulation

Anders Ståhlberg

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Sarcomas characterized by FET (*FUS*, *EWSR1*, *TAF15*) fusion oncogenes (FET sarcomas) contain a hierarchy of different cell types, forming subpopulations of cancer stem cells, proliferating expansion phase cells and differentiated cells. The most common entities of FET sarcomas are Myxoid liposarcoma are Ewing sarcoma. The identity and function of these subpopulations, and the pathways that control transitions between different cell states are essentially unknown in FET sarcomas. To facilitate studies of tumor subpopulations and their dynamic transitions into different cell states we are applying a qPCR based technique to simultaneously interrogate multiple analytes (DNA, mRNA, miRNA, ncRNA and proteins) in the same single-cell. This approach open up new avenues for detailed correlation studies of multiple and different classes of analytes at the single-cell level. Varimax and trend-PCA are new data visualization tools that simplify data interpretations facilitating discoveries. Here, we applied single-cell analysis to show that Myxoid liposarcoma has a dysfunctional cell cycle regulation. In addition, we identified a novel quiescent cell population with distinct expression profile. Importantly, the FET fusion oncogene can be linked to both these tumor characteristics. In this presentation we will also discuss how single-cell based analysis in general may help us to gain new and detailed information about cell states and cell fate decisions.

SMARTer Solutions for Low-Input Transcriptome Sequencing … and more !

François-Xavier Sicot

Takara Clontech, Francois-Xavier.Sicot@takara-clontech.eu

Next-generation sequencing (NGS) has increased our understanding of biology by enabling highly sensitive RNA expression analysis across a wide dynamic range. As NGS applications continue to grow, so does the need for more powerful tools to work with less-than-ideal samples. As the core of the SMARTer kits for transcriptome analysis, Clontech’s patented SMART® technology utilizes the template switching activity of reverse transcriptase to enable researchers to analyze their most challenging samples, such as single cells, low-input RNA, noncoding RNA, and RNA from degraded samples. In particular, single-cell RNA-seq is one of the one of the more difficult, and fastest growing, applications of NGS. The high sensitivity, and dT-primed protocol of the SMARTer Ultra™ Low family has made these kits the industry standard for single-cell analysis. Also the technology has been adapted for Total RNA-seq with stranded information and ligation-free library generation in less than 5 h from limited amount and quality of RNA. Applications of SMART technology are constantly expanding and now include a novel, ligation-free method for generating ChIP (Chromatin Immune Precipitation) sequencing libraries. This seminar is taking a deeper look into the new technologies being developed for single-cell RNA-seq and other sensitive NGS applications.

G&T-seq: Separation and parallel sequencing of the genomes and transcriptomes of single cells

Iain Macaulay[1], Wilfried Haerty[2], Parveen Kumar[3], Yang Li[2], Tim Hu[2], Mabel Teng[1], Niels van der Aa[3], Paul Coupland[1], Chris Ponting[1,2], Thierry Voet[1,3]

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Advances in genome and transcriptome sequencing from single cells now offer a unique perspective from which to investigate cellular heterogeneity in development and disease. Here we present a novel method, *G&T-seq*, which permits simultaneous sequencing of the genome and the transcriptome from the same single cell.

G&T-seq provides whole genome amplified genomic DNA and full-length transcript sequence and with automation, 96 samples can be processed in parallel.

Using cancer cell lines and other models, we are exploring the relationship between DNA copy number and gene expression. From single cells from the breast cancer cell line HCC38 and matched normal control cells, several thousand transcripts were detected per cell, while low coverage genome sequencing demonstrated that copy number variants observed in bulk sequencing were preserved in the genomic analysis of single cells. Integrated analysis of the genome and transcriptome of single cells also allows validation of genomic single nucleotide variants in transcripts, as well as the detection fusion transcripts and their associated genomic rearrangements.

Intracellular molecular gradients within Xenopus laevis oocytes prepare the cell for asymmetric division

Radek Sindelka, Monika Sidova, Mikael Kubista

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Starting from a single fertilized oocyte; the original egg cell, through manifold of divisions a complex organism is developed that has distinct head-tail (bottom-up), left-right and dorsal-ventral (back-belly) asymmetries. One of the main challenges in developmental biology is to understand how and when these asymmetries are generated and how they are controlled. The African clawed frog (*Xenopus laevis*) ideal model for studies of early development thanks to their very large oocytes. We have developed a unique molecular tomography platform based on RT-qPCR, RNA-seq and iTraq UPLC-ESI-MS/MS to measure asymmetric localization of fate determining mRNAs, non-coding RNAs and

proteins within the oocyte and among the early stage blastomeres. We find the first axis, called animal-vegetal, is formed during oogenesis by mRNA and microRNA gradients and determines head - tail organization. First cell division following fertilization producing 2-cell stage embryo forms the left-right, and second cleavage generating 4-cell embryos specifies the dorso-ventral axis.

Optimisation, MIQE & QC strategies in qPCR

Wednesday, 25/Mar/2015:
8:30am - 12:50pm
Session Chair:
Tania Nolan & Afif M. Abdel Nour

Location:
Lecture hall 14

Presentations

Variability of the Reverse Transcription Step: Practical Implications

Stephen Bustin[2,1], Harvinder Dhillon[2], Sara Kirvell[2], Christina Greenwood[2], Michael Parker[2], Gregory Shipley[4,1], Tania Nolan[1,3]
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The conversion of RNA to cDNA using reverse transcription is a necessary first step for many molecular biology applicatons. While RT efficiency is known to be variable, little attention has been paid to the practical implications of the variability. Several mRNA targets were quantified in RNA samples of varying quantity and quality after RT using commercial reverse transcriptases for cDNA synthesis. An analysis of the relative yield of the markers across the samplesdemonstrated that RT efficiency is enyzme, sample and RNA concentration dependent, resulting in variable correlations between markers in the same sample. This variability translated into relative mRNA expression differences that generally varied between 2- and 3- fold, although higher extremes were observed. These data demonstrate that the inherent variability of the technique is sufficient to call into question the validity ofmany published data sets. Variability can be minimised by selecting the appropriate RT enzyme, concentration of RNA and sufficient characterisation of the individual assay.

qScript XLT cDNA SuperMix: overcoming the common pitfalls of cDNA synthesis as applied to relative quantification and RT-qPCR.

David Mark Schuster, Yun Feng
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Despite the introduction of new methods for gene expression quantification, reverse transcription quantitative PCR (RT-qPCR) remains the dominant method for measuring changes in gene expression, providing high accuracy and precision with broad dynamic range. At the heart of this technique is the reverse transcription of RNA into complementary first-strand DNA product. The ready availability of reliable and easy to use kits has made this critical step of the process routine. However, complacency and the lack of attention to this critical portion of the process can be at the peril of robust and reliable data and introduce potential bias that can misleadingly be attributed to differential gene expression.

This talk will examine a variety of factors that influence RT-qPCR and present an advanced first-strand synthesis system that offers significant improvement in the sensitivity and accuracy of RT-qPCR for routine applications as well as the demanding needs of reverse transcription from limited template amounts such as the study of gene expression in single cells.

New tools for quality assessment of RNA in Molecular Diagnostics

Jens Björkman[1], David Švec[1,2], Robert Sjöback[1], Emelie Lott[1], Mikael Kubista[1,2]
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Since the publication of the MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) guidelines in 2009 users' awareness and clients' requests for quality control (QC) in qPCR has dramatically increased. To comply with MIQE professional providers of qPCR services and contract research organizations emphasize the different aspects of quality control in their offerings. TATAA Biocenter co-authored the MIQE- guidelines, was partner in the SPIDIA consortium (www.spidia.org), member of the workgroup drafting the forthcoming ISO guidelines for the pre-analytical steps in molecular diagnostics, and is certified according to ISO 17025. TATAA early identified the need for and importance of stringent quality control in qPCR and has focused on developing methods and tools to asses sample, assay and performance quality parameters.

In this work we have identified biomarkers resistant to nucleolytic RNA degradation that can be used to assess RNA quality in samples exposed to temperatures and conditions at which nucleases are active or freeze-thawing. We also developed protocol to assess physical and chemical degradation, such as damaged caused during tissue fixation. These methods will be presented demonstrating their advantages in terms of sensitivity, performance and ease of use compared to traditional methods such as capillary electrophoresis and the 5'-3' assay.

ThermaStop™ Hot-Start Reagent: Properties and Benefits

Alexandra Isabel Over[1], Lawrence Wangh[2]
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This presentation introduces a novel ThermaStop™ hot-start reagent, developed in collaboration between Biosearch Technologies, Inc. and Professor Lawrence Wangh's laboratory at Brandeis University. The ThermaStop hot-start reagent is an oligonucleotide that can adopt a hairpin conformation. This reagent interacts with DNA polymerase in a temperature-dependent manner, rendering the enzyme more selective against 3' mismatches. As a result, the ThermaStop reagent suppresses the formation of competing, non-specific products, thereby reducing scatter among replicate reactions, and increasing the yield of intended amplicons. ThermaStop hot-start activity rivals or exceeds that of hot-start antibodies. Unlike hot-start antibodies, however, ThermaStop denaturation at high temperatures is reversible. Whenever the reaction temperature is lowered below the primer annealing temperature, the ThermaStop oligo refolds into its active conformation, and thereby provides "cold-stop" protection to amplification products. Thus, the ThermaStop reagent enhances the use of low temperature probes in real-time and end-point asymmetric PCR methods such as LATE-PCR, and also increases the accuracy of downstream applications such as, re-amplification and sequencing. The ThermaStop hot-start reagent is compatible with all PCR strategies because its activity depends only on the functional

ratio of reagent to DNA polymerase. The ThermaStop reagent also displays a unique signature in SYBR Green melting curves that can be used to determine the lowest effective concentration of reagent necessary for a particular reaction. In summary, the ThermaStop is a multifaceted, reversible, hot-start reagent that interacts with DNA polymerase to improve the precision and yield of all primer dependent amplification reactions. The ThermaStop reagent is the first of a family of reagents to address all forms of mis-priming that will be commercially available in the near future.

Engineered DNA Polymerases

Ramon Kranaster
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By combinatorial protein design myPOLS Biotec is evolving new generations of DNA polymerases for advanced applications. As an example, we recently developed HiDi DNA polymerase that is an engineered DNA polymerase developed for approaches in which **hi**gh **di**scrimination between matched and mismatched primers is required (e.g., HLA analysis, allele-specific PCR (ASA) or methylation-specific PCR (MSP)). Whereas many DNA polymerases tolerate mismatched primers at least to some extent, HiDi DNA polymerase efficiently discriminates those and specifically produces amplicons in case of perfectly matched primer pairs only. This renders HiDi DNA polymerase the prime enzyme for SNP detections, HLA genotyping or the analysis of single CpG methylation sites in MSP. Based on HiDi DNA polymerase, we provide custom-made advanced and reliable qPCR mixes. Furthermore, since HiDi DNA polymerase allows allele-specific PCRs directly from oral swabs without requiring DNA isolation or any other tedious sample processing step, a Direct SwabPCR 2x Master Mix was established.

We also developed Volcano DNA polymerase – an engineered, extremely thermostable enzyme that has both, reverse transcriptase and PCR activity. Volcano DNA polymerase has a half-life at 95°C of >40 min and it facilitates “zero-step” RT-PCRs directly from RNA templates (without an isothermal reverse transcription step). These properties allow reverse transcription reactions at high temperatures, thus minimizing the problems encountered with strong secondary structures in RNA that only melt at elevated temperatures.

myPOLS Biotec is actively pursuing research on DNA polymerases to shape their properties for advanced applications. In the next months new DNA polymerases will be introduced by myPOLS Biotec that include:

- DNA polymerases resistant to known inhibitors (e.g., from blood or plants) allowing direct PCR without tedious sample preparations
- DNA polymerases enabling multiplex DNA and RNA diagnostics
- DNA polymerases that are tolerant to high salt conditions
- DNA polymerases allowing PCR from damaged DNA samples
- DNA polymerases that tolerate highly modified nucleotides
- Temperature sensitive DNA polymerases with intrinsic hot start function

Still missing the right DNA polymerase for your applications? We will assist you in establishing your approach using our wide knowledge on DNA polymerase engineering. With our expertise we tailor DNA polymerases for your needs! Contact us!

Reducing time and error rates by automating qPCR workflows

Matjaž Hren[1], Kristina Gruden[2], Klemen Zupančič[1], Nataša Mehle[2], Manca Pirc[1,2], Urška Čepin[1], Maja Ravnikar[2]
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Advanced technologies are being employed in the labs with the main aim to solve problems, improve existing solutions and help achieve the goals set by and for researchers. The major challenges researchers are facing today are: keeping up with the ever-rising workload and data analysis requirements. Lab automation market research conclusions suggest that almost 50% of researchers’ time is still spent on data and information tasks. Therefore, the most dramatic changes are expected to occur in hardware and software technologies and regulatory areas. Due to the overall complexity and non-flexibility of most solutions available today, there is a growing need for smart solutions which combine the ease of use, flexibility and reliability.

A growing challenge in laboratories dealing with high throughput real-time PCR (qPCR) analyses for diagnostic or research purposes is how to make the complete process from sample preparation to data analysis, interpretation and reporting faster without compromising traceability and reliability of the results. We are presenting a case study on detection of bacteria with qPCR in plant samples, showing improvements on two levels: at the level of wet-lab automation and at the level of complete process management using a web-based smart qPCR dedicated set of software tools.

New improvements on sample handling such as automated simple and quick homogenization and automated magnetic bead based DNA extraction method greatly increase the speed of sample processing without compromising the DNA extraction. Additional improvements include facilitation of loading of mastermixes and samples onto qPCR plates using a smart pipetting assistant device. Further on, the use of dedicated software tools automates and reduces the time needed for complex, repetitive, time consuming actions like experiment design, creation of templates for lab work, data analysis, result interpretation and reporting. The full traceability of all researchers’ actions within the qPCR workflow is automatically being saved. All information is being stored in one place in a quality assurance compliant way (ISO 17025). Powerful search enables researchers to instantly find any needed data. Communication with LIMS and different qPCR thermal cyclers is also being established. In addition, external protocols and files including sample images and similar are being easily uploaded and stored. The set of software tools enables lab managers to have complete overview on the work progress in their labs and access to real time lab performance from different locations.

Such modern approach can save up to 40% of time spent on data and information tasks. It reduces error rates and the burden of ever-rising workload on the key lab personnel, which is of extreme importance in case of high throughput qPCR.

Middleware Solutions To Improve Molecular Diagnostics; Connectivity, Quality Control And Lean Processes

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Objectives: Molecular diagnostic laboratories currently have implemented devices from nucleic isolation to real-time detection, including pipetting robots, mostly from different companies to manage their diagnostic processes. With increasing quality control demands (ISO15189, GAMP5), these processes do become critical components in patient management. Our MiddleWare solution FlowG improves lab processes and QC demands.

Methods: Our MiddleWare is linked to a Laboratory Information System using Microsoft Access and Visual Basic for Applications. All processes are analyzed for critical steps, automated and subsequently validated. The software is implemented around our routine laboratory diagnostic set-up.

Results: Three different isolation robots, six pipetting robots and eight ABI7500 devices are linked. All information ont control reagents (internal and positive controls) are used for assay validation. For quantitative laboratory developed assays, values are automatically assigned based on

validated and pre-calculated standard curves. QC data are available in the database to enable time independent evaluation of reagents and controls. The software is validated along the GAMP5 guidelines for risk analysis. Asides routine diagnostics, the FlowG concept is expanded to sequence analysis. These processes are now an integrated part of the software.

Conclusion: Our MiddleWare solution is the first to enable connectivity between devices from different vendors. The implementation is within an ISO-regulated manner and make our diagnostic processes more LEAN. Furthermore, developments towards using this solution for sequencing and QC purposes are implemented and enhances patient care.

Digital publication: how making the MIQE guidelines easier to follow.

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Aim of the MIQE & qPCR iBook -- *"How to apply the MIQE Guidelines – a visual, interactive and practical qPCR guide!"*
The MIQE guideline is becoming more and more popular among researchers due to its strong scientific basis and focus on biological relevance, its adaptability to different research settings and technologies. In order to improve its use, a useful software application (app) for smartphones and tablets has been developed (Abdel Nour et al. 2013). The app has been downloaded thousands of times since their launch in 2011. Today, in addition to these tools, we offer a new digital support -- the MIQE & qPCR iBook.
This new interactive publication aims at helping the research community to understand and use the MIQE goal. The MIQE & qPCR iBook is a fancy visual interactive tool, interfacing scientific publications, with educating pictures, videos and scientific talks. Authors from the academic field as well from industrial research present their qPCR and dPCR applications in the MIQE context and describe it on the basis of educational questionnaires or interactive 'how to do' instruction sheets.
This MIQE & qPCR iBook deliver the MIQE guidelines directly to the researcher and help to solve the problems in quantitative PCR, RT-qPCR, digital PCR, single-cell qPCR, and microRNA applications (... and in more fields of application).
Chapter after chapter, each point of the MIQE guideline and its developments are addressed from both theoretical and practical points of view. We hope you like our explanatory, interactive and educational iBook concept, showing the advantages of the MIQE guidelines in an easy and understandable way, and to guarantee the successful qPCR or dPCR application at the bench.
Please let us know, if you want to participate with an own application and chapter – contact iBook@bioMCC.com

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Non-coding RNAs 2

Wednesday, 25/Mar/2015:
2:00pm - 4:30pm
Session Chair:
Pieter Mestdagh
Robert Sjöback

Location:
Lecture hall 14

Presentations

Decoding lncRNA functions using high-throughput pathway perturbation.

Pieter Mestdagh, Jan Hellemans, Ariane De Ganck, Jo Vandesompele
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Genome-wide studies have shown that our genome is pervasively transcribed, producing a complex pool of coding and non-coding transcripts that shape a cell's transcriptome. Long non-coding RNAs or lncRNAs dominate the non-coding transcriptome and are emerging as key regulatory factors in human disease and development. Still, only a fraction of lncRNAs has been studied experimentally. In order to gain insights in lncRNA functions on a genome-wide scale, we performed high-throughput pathway perturbations followed by total RNA sequencing. Cells were treated with 90 targeted compounds and 90 transcription factor siRNAs, yielding a total of 180 individual perturbations. For each perturbation, differentially expressed lncRNAs were identified and mapped to pathways using matching protein-coding gene expression data. We define a functional context for thousands of lncRNAs that can serve as a starting point to guide more focused experimental studies and accelerate lncRNA research.

New sensitive and specific method for microRNA analysis

Robert Sjöback[1], Lukáš Valihrach[2], Mikael Kubista[1,2]
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MicroRNAs (miRNAs) are small non-coding RNAs that function as important biological regulators in viruses, plants, and animals (1). The single-stranded miRNAs regulate gene expression at the post-transcription level and the dysregulation of miRNAs is associated with various human diseases (2-4). Recent reports show microRNAs are abundant not only in tissues but also in body fluids and they show great potential as minimum-invasive biomarkers in the diagnosis and prognosis of cancers and other diseases (5, 6). However, there are two main challenges when analyzing miRNAs by molecular methods: 1) miRNAs are short, usually not more than 22 nucleotides, which is the length of conventional PCR primers; 2) closely related miRNAs may differ in only a single nucleotide position. Current methods approach these challenges by extending the length of the miRNA using either miRNA specific RT primers or by non-specific RT primers, which compromises specificity and sensitivity (7). Here we present a novel method for the detection and quantification of short nucleic acids that has higher sensitivity than current approaches with concomitant enhanced specificity.

1) He et al., Nat Rev Genetics 2004, 5: 522-31. 2) Esquela-Kerscher A & Slack FJ, Nat. Rev. Cancer 2006, 6: 259-69. 3) Michael et al., Mol. Cancer Res. 2003, 1: 882-91. 4) Dimmeler S & Nicotera P, EMBO Mol. Med. 2013, 5: 180-90. 5) Brase JC et al., Mol. Cancer 2010, 26: 306. 6) Chen et al., Cell Res. 2008, 18: 997-1006. 7) Mestdagh P et al., Nature Methods 2014, 11:809-815.

MiRNA Profiling In Tumor Tissue, Body Fluids And Exosomes - A Combinational Techniques Approach Of NGS And QPCR.

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miRNA has gained a pivotal role in molecular diagnostics and disease analysis. Due its stable nature and an abundant presence either in the tissue of origin, in microparticles or free circulating, it is a viable and interesting analyte. To understand the dynamics and spectrum of this biological dilution - from the production site down to circulation – we analysed glioblastoma tissue in conjunction with cerebrospinal fluid (CSF) and serum from the same patients. As the generation of microvesicles, general gene expression and epigenetic changes (methylation and hydroxyl-methylation of DNA) might mechanistically contribute to the phenomena, these characteristics were also measured. miRNA was

primarily analysed via miRNA-Seq on a HiSeq instrument to allow a certain depth of the data. A data convergence of the different biological levels and source materials in addition to further information via qPCR was gathered. The profiling procedure and results underlining the inherent dynamics will be presented.

Development and Optimisation of PCR Assays to Analyse MicroRNAs and their Target Genes

David Arthur Simpson
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Introduction: Endothelial progenitor cells (EPCs) isolated from blood release microRNA-containing extracellular vesicles (EVs) which can potentially be harnessed to modulate angiogenesis as a treatment for vascular disease. These EVs contribute to the pool of circulating microRNAs, which provide biomarkers for many conditions. Quantification of microRNAs is required both to study their role in vascular repair and to exploit their potential as biomarkers. Multiple issues need to be considered in the design of a successful assay. RT-PCR reagents and template concentrations must be optimised. The final reaction volume must be minimised to reduce reagent use and amount of template required. Increasing the rate of thermal cycling brings time-savings and can be critical for certain clinical applications. The specificity of the assay with regard to microRNA isomiRs must be defined.

Methods: Probe-based assays and polyadenylation followed by oligo-dT primed reverse transcription and PCR with SYBR Green were employed. PCR reactions were set up using an Echo liquid handler (Labcyte), which uses acoustic energy to transfer 25nl droplets. qPCR was performed using a 384 well LightCycler 480 (Roche) or a rapid thermal cycler (xxpress, BJS Biotechnologies). RT-PCR products were sequenced using the Ion Torrent platform (Life Technologies).

Results: The quality of data was maintained as qPCR volumes were reduced to 2 µl. MicroRNAs can be amplified from plasma in less than 10 min using the xxpress cycler. Sequencing of RT-PCR products provides a profile of isomiRs for specific microRNAs comparable to sequencing of entire small RNA libraries.

Conclusion: The ability to accurately transfer nanolitre volumes and therefore adopt very low reaction volumes facilitates rapid optimisation of PCR reaction conditions and saves reagents and template. Characterisation of microRNA isomiRs and rapid detection of them from plasma broadens their potential as clinical biomarkers.

Improved Small RNA Library Preparation Workflow for Next Generation Sequencing

Sabrina Shore, Jordana Henderson, Anton McCaffrey, Gerald Zon, Richard Hogrefe
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Next generation sequencing (NGS) can be used to analyze microRNAs (miRNAs), small non-coding RNAs that are important therapeutic targets and diagnostic markers. Commercially available small RNA sequencing library preparation kits require large inputs (100 ng) and a laborious gel purification step, which is not amenable to automation. Additionally commercial kits are hindered by adapter dimer formation, where 5´ and 3´ adapters ligate without an intervening RNA insert. Adapter dimers preferentially amplify relative to the library during PCR amplification. This is exacerbated at low RNA inputs where adapter dimers can greatly diminish usable sequencing reads. We describe an optimized small RNA library preparation workflow which suppresses adapter dimers, allows for RNA inputs as low as 1 ng and eliminates the need for gel purification. Chemically modified adapters and an optimized protocol were employed to suppress adapter dimers while still allowing for efficient library ligation. Library preparation with modified adapters was compared to the Illumina TruSeq® Small RNA Sample Prep Kit. Non-gel purified samples were purified with the Agencourt® AMPure® XP Kit. Samples were sequenced on an Illumina HiSeq™. Our modified adapter workflow was benchmarked against the TruSeq® Kit at 100 and 10 ng inputs. The modified adapter workflow allows RNA inputs as low as 1 ng and generates less than 1% adapter dimer reads when gel purified (Table 1). In contrast, the TruSeq® Kit yields 14% and 51% adapter dimer reads at 100 and 10 ng inputs, respectively. TriLink's modified adapter workflow with magnetic bead-based size selection yields less than 10% dimer for all input levels, while the TruSeq® Kit results in a minimum of 14% dimer reads at the highest input level. TriLink's modified adapter workflow improves small RNA library preparation by significantly reducing adapter dimer formation. In fact, our improvements allow for sequencing from 1 ng of total RNA without compromising valuable sequencing reads, which was previously not feasible. TriLink's workflow with magnetic bead-based size selection, an automatable technique, results in lower amounts of dimer reads than current methods using gel purification. Replacement of gel purification with an automatable purification step results in less hands-on time, better reproducibility and higher throughput. The modified adapter workflow surpasses other currently available technologies and provides significant improvement to small RNA NGS.

qPCR BioStatistics & BioInformatics

Wednesday, 25/Mar/2015:
8:30am - 12:30pm
Session Chair:
Jan Ruijter & Stefan Rödiger

Location:
Lecture hall 15

Presentations

Removal of Between-Plate Variation in qPCR with Factor Correction: Completion of the Analysis Pipeline Supported by RDML.

Jan Ruijter[1], Jan Hellemans[2], Adrian Ruiz-Villalba[1], Maurice Van Den Hoff[1], Andreas Untergasser[3]
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Quantitative PCR is the method of choice in gene expression analysis. However, the number of experimental conditions, target genes and technical replicates quickly exceeds the capacity of the qPCR machines. Statistical analysis of the resulting data then requires the correction of between-plate variation. Application of calibrator samples, with replicate measurements distributed over the plates assumes a multiplicative difference between plates. However, random and technical errors in these calibrators will propagate to all samples on the plate. To avoid this effect, the systematic bias can better be corrected when there is a maximal overlap between plates using Factor Correction [Ruijter et al. Retrovirology, 2006]. The original Factor Correction program is based on Excel input and calculates corrected target quantities. To implement this correction into the analysis pipeline from raw data through LinRegPCR into qbase-plus, a new version of the program was created to handle RDML files. This version saves the corrected N0 values as efficiency-corrected Cq values to be used in further calculations. This program thus completes the analysis pipeline of qPCR data supported by RDML.

Unexpected System-specific Periodicity In Quantitative Real-Time Polymerase Chain Reaction Data And Its Impact On Quantification

Andrej-Nikolai Spiess[1], Stefan Rödiger[2], Thomas Volksdorf[3], Joel Tellinghuisen[4]

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The "baseline noise" of quantitative real-time PCR (qPCR) data is a feature of every qPCR curve and has substantial impact on quantitation. In principle, two different forms of baseline noise can be encountered: (i) the dispersion of fluorescence values in the first few cycles of a qPCR curve around their mean (within-sample noise) and (ii) the dispersion of fluorescence values between different qPCR curves at the same cycle (between-sample noise). The most predominant effect that results in between-sample noise is an overall shifting of the qPCR curve on the y-axis ("baseline shift"), which is frequently compensated by "baselining" qPCR data. Common approaches are to subtract an averaged (Lievens *et al*., 2012; Rutledge, 2011; Goll *et al*., 2006), iteratively estimated (Ramakers *et al*., 2003; Ruijter *et al*., 2009) or lower asymptote derived (Tichopad *et al*, 2003; Peirson *et al*., 2003; Spiess *et al*., 2008) baseline value from all fluorescence values prior to quantitation (compare Table 1 in Ruijter *et al*., 2013).

Recently, we showed preliminary results on a published large scale technical replicate dataset (Ruijter *et al*., 2013) that indicated between-sample periodicity for fluorescence values at early and late cycle numbers (Tellinghuisen & Spiess, 2014). A more detailed interrogation of the between-run noise periodicity revealed that this effect occurs at all cycle numbers and constitutes a second and completely independent noise component that adds to the overall baseline shift. Most importantly, periodic noise persists even after classical "baselining" and results in a propagation of periodicity into estimated *Cq* values when using fixed threshold methods (LinReg, FPKM, DART, FPLM), hence resulting in periodic *Cq* values. In contrast, *Cq* values obtained from variable threshold methods based on first- or second-derivative maxima (Cy0, Miner, 5PSM) or from normalization of fluorescence data are completely devoid of periodic noise, corroborating the feasibility of these approaches.

The origin of periodic noise in qPCR data remains elusive. By employing a larger cohort of published and also self-generated high-replicate qPCR data from different platforms, we used classical algorithms of time series/signal analysis (i.e. autocorrelation analysis) to characterize the periodicity in more detail. Interestingly, we generally observed a periodicity of 24/12 for 384/96-well plate systems, respectively. These findings strongly suggest an effect of uneven temperature profiles in peltier block systems or variable liquid deposition of manual/automated multichannel pipetting systems, manifesting themselves as periodic qPCR data. We will present ways to eliminate periodic noise from qPCR data that results in a more reliable estimation of *Cq* values.

The PrimerBank database: an analysis of high-throughput primer validation.

Athanasia Spandidos[1,2,3], Xiaowei Wang[1,2,4], Huajun Wang[1,2], Brian Seed[1,2]

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qPCR remains the gold standard used for validation of gene expression measurements from high-throughput methods such as DNA microarrays, however, non-specific amplification is frequently an issue. In order to overcome this, we developed the PrimerBank database, a public resource containing primers that can be used under stringent and allele-invariant amplification conditions. PrimerBank can be used for the retrieval of human and mouse primer pairs for gene expression analysis by PCR and RT-qPCR. Currently, the database contains 497,156 primers which cover 17,076 and 18,086 genes for the human and mouse species, respectively, corresponding to around 94% of all known protein-coding gene sequences. PrimerBank also contains information on these primers such as T_m, location on the transcript and expected PCR product size. Primer pairs covering most known mouse genes have been experimentally validated by amplification plot, gel electrophoresis, DNA sequence and thermal denaturation profile analysis, and all the experimental validation information together with primer information can be freely retrieved from the PrimerBank website (http://pga.mgh.harvard.edu/primerbank/). The database can be searched using various search terms. One of the advantages of PrimerBank primers is that they have been designed to work under a common PCR thermal profile, so they can be used for high-throughput RT-qPCR in parallel or genome-wide RT-qPCR. The expression profiles of thousands of genes can be determined simultaneously using high-throughput platforms available, making PrimerBank primers useful for gene expression analysis on a genome-wide scale.

Occurrence of unexpected PCR artefacts warrants thorough quality control

Adrián Ruiz-Vilalba[1], Bep van Pelt-Verkuil[2], Quinn Gunst[1], Maurice van den Hoff[1], Jan Ruijter[1]

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A recent comparison of qPCR data analysis methods showed that some of the amplification curve analysis methods perform better than the classic standard curve and Cq approach on indicators like variability and sensitivity. In this comparison the possibility to characterize the amplification curve and thus assess its quality remained under-exposed. To this end, different datasets have been compared. Our results show that for a significant fraction of the genes, low initial target concentrations lead to the amplification of artifacts independently of the primer specificity. These non-specific amplification curves are indistinguishable from those resulting in the correct products; they show similar baseline, PCR efficiency and plateau fluorescence behaviours. The validation of specific amplification curves requires a quality control in which the design of the plate, a melting curve analysis (MCA) and electrophoresis gels are combined. In addition, our data suggest that the relative concentration of the template in the cDNA input and of the primers determines the appearance of the PCR artifacts. Unexpectedly, the presence of non-template foreign cDNA seems to be an essential requirement for the amplification of the correct specific qPCR target.

Impact of Smoothing on Parameter Estimation inQuantitative DNA Amplification Experiments

Stefan Rödiger[1], Andrej-Nikolai Spiess[2], Michał Burdukiewicz[3]

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Quantitative real-time polymerase chain reaction (qPCR) is one of the most precise DNA quantification methods. The parameters quantification cycle (*Cq*) and amplification efficiency (*AE*) are commonly calculated from distinct location indices of the amplification curve (threshold fluorescence, first- or second-derivative maxima) to quantify qPCR reactions. Consequently, a precise analysis is the requirement to quantify the copy number in samples [1]. Several smoother and filter methods for minimizing inherent noise in qPCR data have been proposed in the peer-review literature. Despite the fact that smoothing steps are so frequently employed during amplification curve analysis and generally taken for granted, the question that arises is if should we really accept to use any of these methods without paying attention to their possible implications.

The smoothers and filters we compared in our investigation are widely used to compensate for noisy data. We found no fundamental controversy in the scientific community about the smoothers and filters used in our study. All of them are thoroughly tested, peer-reviewed, and well accepted. In our study we specifically addressed the question, which of the smoothers is appropriate for amplification curve data acquired by isothermal amplification or qPCR.

Due to the lack of comprehensive models we have chosen an empirical approach in combination with amplification curve simulation to evaluate the smoother and filter functions in a testable scenario. For this purpose, we analyzed the impact of the smoother methods on real-world data from different thermo cycler equipment (low through-put and high-throughput cyclers) as well as different amplification methods. We also used in our analysis "user-controlled" noise structures based on Monte Carlo simulations.

Our results indicate that selected smoothing algorithms affect the estimation of *Cq* and *AE* considerably. The commonly employed moving average filter performed worst in all qPCR scenarios. Least bias was observed for the Savitzky-Golay smoother, Cubic Splines and Whittaker smoother. In general, we found a low sensitivity to differences in *AE*, whereas other smoothers like Running Mean introduced a significant *AE* dependent bias. We developed open source software packages to facilitate the selection of smoothing algorithms that can be incorporated in an analysis pipeline of qPCR experiments. The findings of our study were implemented in the R packages *chipPCR* and *qpcR* [2,3], freely available from "The Comprehensive R Archive Network". We anticipate that our findings serve as guidelines for the selection of an appropriate smoothing algorithm in diagnostic qPCR applications. However, a general feasibility of qPCR data smoothing remains to be demonstrated.

[1] Pabinger and Rödiger et al., *Biomolecular Detection and Quantification* (2014), 1/1, 23-33. [2] Spiess AN et al., *Clinical Chemistry* (2015), preprint. [3] Rödiger et al. (under revision), *Bioinformatics* (Oxf.)

RDML qPCR Data Format - Ready For The Next Level?

Andreas Untergasser[1], Steve Lefever[2], Jasper Anckaert[2], Jan M Ruijter[3], Jan Hellemans[4], Jo Vandesompele[2,4]
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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). 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 (Lefever et al., NAR). 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.

Today, several instrument manufacturers realized its potential and implemented functionality to export data in the RDML-format. Third party software (LinRegPCR and qbasePLUS) uses this information for advanced data analysis. Due to the flexibility of RDML, the majority of the current software uses only parts of the format. Furthermore, with different RDML versions available, the need to convert between versions became obvious. The open source editor RDML-Ninja was designed to edit RDML-files and convert between different versions (sourceforge.net/projects/qpcr-ninja/). It should serve as reference implementation of the RDML-format and assist researchers, reviewers as well as software developers by offering access to all data in an RDML-file.

Ultimately, RDML could be extended to store all information required by MIQE. Currently the information required by MIQE seems overwhelming to a researcher, but RDML offers an easy way out. All the information would be only entered once and stored in a basic RDML file. Researchers would not have to re-enter this information with every qPCR run, but will import from this RDML file only the parts needed for the current qPCR run. Furthermore integration of MIQE in RDML and RDML-Ninja would allow checking to which extend MIQE information is provided by calculating the checklist completeness based on a provided RDML-file. We would like to discuss this vision, its chances and its applicability.

RDML Consortium Meeting

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Molecular Diagnostics in Agriculture, Veterinary Medicine, Food & Environmental Science

Wednesday, 25/Mar/2015:
2:00pm - 4:30pm
Session Chair:
Irmgard Riedmaier-Sprenzel & Michael W. Pfaffl

Location:
Lecture hall 15

Presentations

Comparison of Different RNA Sources to Examine the Lactating Bovine Mammary Gland Transcriptome using RNA-Sequencing

Angela Canovas[1], Claudia Bevilacqua[2], Gonzalo Rincon[1], Pauline Brenaut[2], Alma Islas-Trejo[1], Russell C. Hovey[1], Marion Boutinaud[3], Caroline Morgenthaler[2], Monica K. VanKlompenberg[1], Juan F. Medrano[1], Patrice D. Martin[2]
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The objective of the present study was to examine five different sources of RNA, namely mammary gland tissue (MGT), milk somatic cells (mSC), antibody-captured milk mammary epithelial cells (mMEC), milk fat globules (mFG) and laser microdissected mammary epithelial cells (LCMEC), to analyze the bovine mammary gland transcriptome, using RNA-Sequencing. Given the small amount of materials we started from, especially from mFG, mMEC and LCMEC, the five RNA preparations were amplified, using the Ribo-SPIA technology from the Ovation RNA-seq System (NuGEN, San Carlos, CA). Our results provide an objective assessment between invasive and non-invasive sampling methods to

analyze and compare the transcriptome of mammary gland tissue and milk cells. This information is of value to choose the most appropriate sampling method for different research applications, to study specific physiological and health states during lactation. The simplest procedures to study the transcriptome associated with milk appears to be the isolation of total RNA directly from mSC or mFG from milk. Our results indicate that the mSC and mFG transcriptomes are representative of MGT and LCMEC, respectively, and can be used as effective and alternative samples to study mammary gland expression without the need to perform any tissue biopsy.

Optimized library preparation for sequencing of whole bacterial genomes and low density microbiota by Illumina-based NGS

Mareike Wenning, Christopher Huptas, Manuela Schreiner, Siegfried Scherer

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Next-Generation Sequencing (NGS) technologies have paved the way for rapid and cost-efficient analyses of transcriptomes, microbiomes as well as de novo sequencing and re-sequencing of genomes. Library preparation is a crucial step in the generation of high quality data and may have a major impact on the success of data analysis and interpretability of data. Difficulties and improvements of two different sample preparation procedures are presented.

With the introduction of PCR-free library preparation procedures (LPPs) for de novo genome sequencing major improvements were made in comparison to the initial PCR containing LPPs, as PCR biases are largely reduced. In this study modified versions of the widely used Illumina TruSeq® DNA PCR-free library preparation protocol were developed that enable the generation of sequencing libraries with longer average insert sizes leading to substantial assembly improvements using SPAdes, which is currently one of the best performing assemblers with regard to bacterial de novo genome assembly. Through the introduced modifications, DNA quantitation by qPCR can be omitted and fewer reagents are consumed. Furthermore, the relationships between genomic GC content, average library insert size and sequencing quality were investigated.

For analyzing the biodiversity of microbiota, PCR is an indispensable step, as it is needed to amplify a fragment of the 16S rRNA gene, which is sequenced subsequently. Here, the extraction of DNA and use of adequate PCR conditions are of utmost importance. This is particularly true for microbial communities with low cell counts in difficult matrices. Raw milk microbiota are of high interest, but raw milk contains high fat and protein contents as well as high amounts of accompanying eukaryotic DNA originating from the cow's somatic cells. We have developed a protocol for DNA extraction minimizing the content of somatic DNA and have performed different PCR strategies such as droplet digital PCR for analysing possible PCR bias. The data obtained show that DNA extraction out of low density communities requires substantial effort, but sequencing of such microbiota is possible.

Transcriptomic Biomarkers in Food Safety: RNA Biomarkers against the abuse of growth promoters

Irmgard Riedmaier-Sprenzel, Melanie Spornraft, Michael, W. Pfaffl

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The use of growth promoting agents in animal husbandry is strictly forbidden the European Union. This ban is controlled within a strict control plan where residues of known growth promoting agents are identified using chromatographical methods in combination with mass spectrometry or may be screened by immuno assays. New designed xenobiotic agents or new modes of application, e.g. the administration of substance cocktails are not detectable with those methods. To ensure an efficient tracing of misused anabolic agents, new detection methods have to be developed. One promising way is to monitor the physiological effects of the given substances on the molecular level. The analysis of the transcriptome has already been shown to be a promising approach to detect the pharmacological action of a substance in different organs and matrices.

In a pilot study, RNA-Sequencing technology was used to screen for changes in the transcriptome of bovine liver caused by treatment with steroid hormones. Thereby a first biomarker pattern could be identified that enabled the separation of treated animals versus untreated animals using biostatistical clustering methods.

In order to test the drug dependence of such biomarkers, the identified biomarker candidates were validated in livers of veal calves treated with the b-agonist clenbuterol or another steroid hormone implant, respectively. Using the dynamic principal components analysis (PCA) algorithm, a biomarker signature could be detected that allowed the discrimination of treated and untreated individuals.

High throughput sequencing was also used to screen for additional biomarker candidates on mRNA and small RNA level in other target tissues. Those results indicate a high potential of transcriptomic biomarkers for the development of a new screening method that is independent of the given drug.

Challenges in Detection of Genetically Modified Organisms

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Genetically modified organisms (GMOs) are organisms in which the genetic material has been altered through the application of gene technology in a way that does not occur naturally through mating and/or natural recombination. There are considerable differences between countries in the adoption of this technology therefore labelling requirements have been set-up in many countries to facilitate international trade and to provide information to consumers. At present, quantitative real-time polymerase chain reaction (qPCR) is the most commonly accepted and used method for detection, identification and quantification of GMOs and considerable efforts have been invested in the understanding and critical evaluation of this technology. Nevertheless, there are challenges that should still be highlighted, such as inhibitors commonly present in different matrices, possible sequence mismatches, characteristics of taxon-specific genes and the quality of the reference materials, as these remain potential sources of measurement uncertainty. In addition, with steadily increasing number of GMOs developed and approved worldwide, the present qPCR methodology may no longer be fully suited to purpose. Several multiplex qPCR methods has already been developed and new approaches, such as digital PCR (dPCR), are being investigated.

A Transcriptomic Approach To Ascertain Insights Into The Etiology Of Brown Trout Syndrome

Marc Young[1], Jens-Eike Taeubert[1], Juergen Geist[2], Michael Pfaffl[3], Ralph Kuehn[1]

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The brown trout syndrome (BTS) is an annually reoccurring disease that causes species-specific die-off of *Salmo trutta* in BTS-impacted pre-alpine rivers of Europe. Based on experimental evidence to date it is hypothesized that BTS is caused by a yet unidentified pathogen. To validate this working hypothesis, as well as to discern the type of pathogen likely to be responsible for BTS, gene expression analyses were conducted on *Salmo trutta* suffering from BTS in order to identify and investigate pathogen-specific immune responses during the progression of the disease. *Salmo trutta* obtained from a single source were held in flow-through tanks supplied with river water at two separate geographic locations (BTS-impacted river section / treatment group; Non-impacted river section / control group) along the same river for a duration of 98 days, with 3 individuals being sampled from each tank (treatment and control) in 7 day intervals (total 14 time points). In a first step, cDNA microarrays were employed in order to screen for regulated pathogen-specific immune responses. Spot identification, intensity quantification and quality control were carried out with the GenePix® Pro 6 software. Analysis of the microarray data were conducted using the open source R software package Linear Model for Microarray Data. Microarray data were background-subtracted using the Kooperberg model-based background correction function, normalized within arrays with the Loess method followed by normalization between arrays using the scale method. Linear models were fitted to the expression data and moderated t-statistics were computed using the empirical eBayes method. Features with a p-value ≤ 0.001 and log 2 -fold change ≥ 1 were deemed significant. The microarray analysis revealed that *Salmo trutta* suffering from BTS exhibit increased hepatic expression of important anti-viral genes. Subsequently in a second step relative gene expression for selected set of anti-viral genes were measured by RT-qPCR in order to construct more concise temporal gene expression patterns for anti-viral response genes in the liver of BTS-afflicted *Salmo trutta* over the course of the disease. The online Primer3 tool was used to design primers for selected anti-viral target genes as well as for three candidate reference genes. The web based comprehensive tool RefFinder identified Ubiquitin to be the most stable candidate reference gene. The ΔΔCt method without efficiency correction as described by Pfaffl was used to determine the relative levels of mRNA expression for the anti-viral target genes normalized to the expression values of the reference gene Ubiquitin between treatment and control group for each time point. Both the microarray and the RT-qPCR analysis reveal that *Salmo trutta* suffering from BTS up-regulate different anti-viral genes throughout the progression of the BTS. Overall the results from the gene expression analyses suggest that BTS is caused by an unknown pathogenic virus.

Poster Session Abstracts

Next Generation Sequencing (Poster 01 – 16)

Potential of Next Generation Sequencing Technology in Molecular Diagnostics – High-Throughput Analysis of the Human Mitochondrial Genome

Agnieszka Piotrowska[1], Ewa Kosior-Jarecka[2], Tomasz Żarnowski[2], Rafał Płoski[3], Katarzyna Tońska[1], Ewa Bartnik[1,4]

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Human mitochondrial DNA (mtDNA) is a small organellar genome, but its variants have a profound effect on cell physiology and thus can be involved in the development of many human diseases. They have been reported to associate not only with mitochondrial diseases – a clinically heterogeneous group of neuromuscular disorders – but also with neurodegenerative disorders, cancers, aging and some complex lifestyle diseases, such as diabetes, obesity and atherosclerosis. mtDNA is highly polymorphic and exists in many copies in the cell, what can lead to heteroplasmy, a situation when molecules with different sequences coexist with each other. Thus a method allowing efficient and precise analysis of the whole mitochondrial genome is required in molecular diagnostics of human diseases. Here we present the results of verification of next generation sequencing (NGS) applicability to whole mtDNA analysis. We also present the preliminary results of mtDNA analysis obtained with the elaborated NGS method in a group of Polish patients with a mitochondrial disorder, glaucoma or a lifestyle disease (diabetes, atherosclerosis and obesity).

Whole mtDNA was enriched from total DNA samples using long-range PCR methods and processed into sequencing libraries accordingly to Illumina protocols. Illumina NGS technology and the MiSeq platform were used for sequencing. Secondary sequencing data analysis was performed using CLC Genomics Workbench bioinformatic software and the identified mtDNA variants were interpreted based on their frequencies and reports in databases and the literature.

High-throughput properties of NGS allowed deep coverage of the mitochondrial genome in the analyzed samples, which is essential for precise determination of the heteroplasmy level of mtDNA variants. However, uneven coverage across the sequence was observed – bias in read depth profiles was similar across different samples, suggesting a problem in the used methodology. Such coverage bias hindered reliable detection of variants with low heteroplasmy levels in regions with low coverage (distinction from sequencing errors) or providing analysis of mtDNA deletion extent, level and breakpoints. Illumina sequencing technology by synthesis (reversible termination of polymerization) proved suitable for sequencing of “problematic” regions present in mtDNA, such as homopolymers, GC-rich regions and short tandem repeats. Novel and rare mtDNA variants were carefully evaluated and classified in terms of clinical significance using *in silico* predictive algorithms and mtDNA databases with established parameters.

We conclude that NGS is a powerful tool and desirable method for comprehensive mtDNA analysis, however, according to our experience, some methodology improvement is needed for proper and reliable molecular diagnostics of human diseases involving mtDNA pathology, such as low heteroplasmy mutations and mtDNA deletions.

RNA-sequencing as a diagnostic method for infectious diseases

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Infectious diseases are caused by invading pathogens like virus, bacteria, fungi or parasites and can be devastating, and sometimes fatal, to the host. To be able to choose the correct treatment against the infection, it is necessary to identify the infectious agent. A correct treatment will increase the possibility to recover from the infectious disease and the care will be more cost effective. Many different methods are used in infection diagnosis, however sometime they fail to identify the agent. High-throughput sequencing offers the advantage of higher sensitivity and the potential to detect a spectrum of microbes. RNA isolated from clinical samples from infected patients contains some expressed pathogen RNA. We evaluated RNA sequencing as a metagenomics-based strategy to uncover known but unexpected pathogen in clinical samples and developed an automated data analysis pipeline.

RNA was purified from several blood or cerebrospinal fluid samples and subjected to RNA sequencing using the Ion total RNA-seq kit for AB Library Builder system and the Ion PGM IC 200 kit for the Ion Chef system and PGM system (Ion Torrent, Thermo Fisher). Totally around 5 millions reads were obtained from each sequencing. The RNA-seq reads were then clustered. From each cluster a representative sequence was blasted to databases. Firstly, to human rRNA and EST databases to exclude human RNA. The rest of the reads were then blasted to virus, bacteria and NT databases (NCBI) and the hits were collected, sorted and further evaluated by medical experts to identify the most likely candidate(s) for the infection. The automated data analysis pipeline was done within 30 min and comprised by a series of scripts and bioinformatic tools.

In conclusion, we have developed a fast laboratory- and bioinformatic pipeline to diagnose infectious diseases with unknown agent within 24 to 32 hours. By using this method we were able to identify otherwise undiagnosed pathogens in the patients.

An Improved cDNA Library Generation Protocol for Transcriptome Analysis from a Single Cell

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As Next Generation Sequencing (NGS) technologies and transcriptome profiling using NGS mature, they are increasingly being used for more sensitive applications that have only limited sample availability. The ability to analyze the transcriptome of a single cell consistently and meaningfully has only recently been realized. SMART™technology is a powerful method for cDNA synthesis that enables library preparation from very small amounts of starting material. Indeed, the SMARTer® Ultra™ Low RNA method allows researchers to readily obtain high quality data from a single cell or 10 pg of total RNA—the approximate amount of total RNA in a single cell. Recent studies have used this method to investigate heterogeneity among individual cells based on RNA expression patterns. A new SMARTer Ultra Low kit has been developed that is simpler and faster while improving the quality and yield of the cDNA produced. The full-length cDNA from this method may be used as a template for library sample preparation for Ion Torrent and Illumina® NGS platforms. Sequencing results for libraries created from single cells or from equivalent amounts of total RNA demonstrate that approximately 90% of the reads map to RefSeq, less than 0.5% of the total reads map to rRNA, and the average transcript coverage is uniform. Improvements in the protocol following first strand synthesis and during cDNA amplification show higher sensitivity with an increase in gene counts and improved representation from GC-rich genes. These data indicate that the improved SMART cDNA protocol is an ideal choice for single cell transcriptome analysis.

Detection of Blood Storage on the Basis of miRNA Expression Pattern as a First Step to Detect Autologous Blood Doping

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Blood Doping is a procedure that uses methods and substances to increase the red blood cell (RBC) mass and to increase the uptake, the transport, and the delivery of oxygen from the lungs to the exercising muscles. This enhances the athlete's performance (by 8-10%) and, therefore, it has been prohibited by WADA (World Anti-Doping Agency) since 1986. There are three types of blood doping: blood transfusion (homologous or autologous), injection with erythropoietin (EPO), and injection of synthetic oxygen carriers. While EPO and synthetic oxygen carriers can be detected with mass spectrometry (MS), homologous blood doping is detectable by measuring different red blood cell populations using flow cytometry. Unfortunately there is still no reliable method to detect autologous blood doping.

The essential step to perform autologous blood doping is to store the RBCs in blood bags for several weeks. During this time span the RBCs are surrounded by a preservative solution. Therefore it is expected that the RBCs respond to these physiological changes in a measurable way.

MicroRNAs (miRNA) are small regulatory RNAs and are already known as helpful biomarkers in several diseases like cancer or diabetes. Thus, it should first be investigated whether it is possible to detect, on the basis of miRNA expression pattern, that the blood has been stored.

To verify this hypothesis, blood samples were taken from 20 healthy subjects before the blood donation and from the blood bags at several points in time during blood storage. The samples from the first six subjects were investigated using miRNA Sequencing, which has the advantage of detecting all existing miRNA in a sample. Using multivariate data analysis tools, it could be shown that blood storage affects the miRNAs profile. Two different clusters could be recognized in the fresh blood samples taken from the subjects and the samples taken from the stored blood bags. This is the first hint that miRNAs react to the addition of a preservative solution that is required for autologous blood doping.

Streamlining NGS workflows by the application of the DNA Integrity Number (DIN) from the Genomic DNA ScreenTape Assay

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The results of numerous molecular screening and assay methods often rely on the overall quality of the genomic DNA (gDNA) input material. However extraction of genetic material can be challenging and often results in low amounts or variable quality of gDNA samples, which are further subjected to time and cost intensive downstream applications. It is therefore widely recommended to perform an initial quality control (QC) of the input material. In order to provide an objective and automated measure to standardize the gDNA integrity assessment, a software algorithm has been developed. This functionality of the 2200 TapeStation system provides a numerical determination of the gDNA integrity and is referred to as the DNA Integrity Number (DIN). Here we show that the initial sample integrity has a huge impact on qPCR downstream applications. Furthermore, especially Next Generation Sequencing (NGS) experiments require intact, high quality gDNA to ensure unambiguous results as only the final step of the workflow reveals if meaningful results have been achieved. This study also demonstrates how DIN obtained by the upfront QC of gDNA on the Agilent Genomic DNA ScreenTape assay has allowed for significant saving of sequencing and sample preparation overhead using FFPE samples in NGS workflows.

DNA Methylation Patterns in Pseudorabies Virus Identified by Short-Read and Single-Molecule Sequencing Methods.

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Pseudorabies Virus (PRV, Suid Herpesvirus 1) is a neurotrophic alpha-herpesvirus infecting swine populations and causing economic losses worldwide. PRV is also utilized for studying the molecular pathomechanism of herpesviruses, as a tract-tracing tool for mapping neuronal circuitries, and for the delivery of genetically encoded fluorescent activity markers. The members of *Alphaherpesviridae* share a life cycle in which the lytic infection of epithelial cells is followed by the establishment of latency in neuronal ganglia. A well-regulated gene expression cascade is characteristic of the lytic phase, where immediate-early, early and late genes are transcribed in strict spatio-temporal order. Mutant strains lacking key transactivators show markedly different infection mechanisms and altered gene expression. Previous studies suggest that viral DNA methylation is present only during the latent phase, when only expression of the latency-associated long non-coding RNA, LAT can be detected. We examined wild type and mutant strains of PRV Kaplan in order to asses viral methylation patterns during the lytic life cycle by

combining bisulfite-converted short-read analysis and single-molecule real-time (SMRT) sequencing. DNA methylation was indeed found in low levels throughout the viral genome, excluding intergenic repetitive regions. DNA methylation in these loci is inversely correlated with actively transcribed regions during infection, indicating the role of genome arrangement in viral gene expression regulation.

The Mix^2 Model Leads To Significantly Improved Accuracy In Transcript Concentration Estimates And Detection Of Differential Expression In RNA-Seq Data

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Quantification of gene isoforms with RNA-Seq is inaccurate due to varying positional coverage bias. Here we propose a statistical model for RNA-Seq data, the Mix^2 model (rd. “mixquare”), which represents this bias by mixtures of probability distributions. The parameters of the Mix^2 model can be efficiently trained with the EM algorithm yielding simultaneous estimates for the relative abundance of gene isoforms and the positional coverage bias.

We initially evaluated the Mix^2 model on synthetic data with 5’ and/or 3’ bias as well as a uniform distribution of the fragment start sites. These tests served to optimize the number of mixture components of the Mix^2 model, which was found to be relatively small. Hence the optimal Mix^2 model is of low complexity and its parameter estimation is therefore fast. In comparison to Cufflinks with bias correction we achieve on real RNA-Seq data a reduction in run-time by up to a factor of 60. Evaluation on our synthetic data, furthermore, highlighted a significant reduction in L^1 distance between true and estimated relative abundances of 50.1% and 65.6% for Cufflinks and PennSeq, respectively.

We evaluated the Mix^2 model on the Universal Human Reference (UHR) and Brain (HBR) sample of the Microarray Quality Control (MAQC) data. Comparing the correlation between qPCR and FPKM values to that of Cufflinks and PennSeq we obtain an increase from 0.44 to 0.6 and from 0.34 to 0.54 in R^2 value for UHR and HBR, respectively. Furthermore, the detection of differential expression between UHR and HBR, based on the FPKM fold change, yields for the Mix^2 model an increase in true positive rate from 0.44 to 0.71 at a false positive rate of 0.1. Clustering the positional coverage bias learned by the Mix^2 model exhibits 6 dominant bias types in the MAQC data, which cannot be detected by other methods. Some of these biases can be explained by the quality of the input RNA and properties of the sample preparation. However, the source of other bias types remains unknown.

We further used the Association of Biomolecular Resources Facility (ABRF) RNA-Seq data to evaluate the correlation of concentration estimates by the Mix^2 Model between different sequencing facilities, library preparations and RNA degradations. These experiments show that the concentration estimates of the Mix^2 model across different conditions are much better comparable than those by Cufflinks. In particular, for all 51 tested comparisons we achieve an increase in R^2 value of between 15% and 30%.

Our results suggest that in comparison to state-of-the-art methods the Mix^2 model yields substantially improved concentration estimates of gene isoforms from RNA-Seq data and leads therefore to higher accuracy in the detection of differential expression. In addition, concentration estimation with the Mix^2 model is substantially faster than that of other methods and leads to better comparability between different sequencing facilities and library preparations.

Insights by Next-Generation Small RNA Sequencing: Comparison of miRNAs and piRNAs in Bovine Blood and Plasma

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Next-Generation Sequencing (NGS) is seen as the state-of-the-art technology in massive parallel sequencing and is considered to be the “gold standard” method of integrated gene quantification in the near future. The investigation of small RNAs via small RNA sequencing (small RNA-Seq) has recently become a new approach in biomarker identification. Especially circulating small RNAs in body fluids are traded as novel goldmine in finding biomarkers which are able to detect diverse pathological conditions, as specimen sampling is non- or minimal invasive and small RNAs exhibit very robust characteristics regarding stability.

An optimized RNA isolation method combined with small-RNA Seq was used to profile microRNAs (miRNAs) and a very emerging new class of small RNAs, the piwi-interacting RNAs (piRNAs), in bovine plasma and whole blood. The presented data evaluation pipeline offered to analyze data regarding quality and allowed annotation and generation of readcount tables by successive reduction of complexity of data sets and aligning sequencing reads to reference databases.

Evaluating annotated readcount data gave information about quantity of the investigated small RNAs in both bio fluids. The ten top-placed miRNAs and piRNAs were highlighted and compared between the screened body fluids. Additionally, a comparison between matching miRNA and piRNA pairs in plasma and whole blood revealed that several miRNAs as well as piRNAs were present at higher levels in plasma than in blood. Especially one miRNA (miR-122) rose evidence that the plasma small RNA signature is not only influenced by RNAs originating from blood cells, as this miRNA is liver-specific.

By investigating the miRNomes and piRNomes, data reinforced the assumption that the circulating small RNA signature in plasma is not only influenced by haemolysis and certain small RNAs could originate from other sources than cellular blood components. Quantifying, profiling and comparing the small RNA footprints in these bio fluids can lead to a better understanding of the role of miRNAs and piRNAs in the circulatory system and lays a basis for future comparative screening projects.

Culture Independent Next Generation Sequencing Based Diagnosis of Blood Stream Infection.

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Objective: Sepsis, a systemic inflammatory response caused by infection, is a serious medical condition causing more than 400,000 deaths in Europe and US every year. Mortality rate rises significantly with delays in initiation of appropriate antimicrobial therapy, therefore rapid and accurate diagnosis is crucial. Traditional culture based methods for diagnosing sepsis/blood stream infection (BSI) have long turnaround times and poor clinical sensitivity. Current molecular methods, all based on PCR, are rapid and accurate but are not comprehensive, seeking only pre-set targets. We have used next generation sequencing (NGS) to overcome the shortcomings of culture and PCR-based diagnosis.

Methods: We have developed a pathogen DNA enrichment strategy capable of removing the vast quantity of human DNA in a blood sample without significant loss of bacteria DNA. This was achieved by immunomagnetic separation (IMS) of leucocytes using anti-CD45 labelled paramagnetic beads followed by differential lysis of the remaining leukocytes and DNase digestion of the human DNA. DNA was then extracted from the remaining pathogens, if present, and the sample was whole-genome amplified and sequenced on theIllumina MiSeq.

Results: A total of 8 blood samples from patients in the Norfolk and Norwich University Hospital intensive care unit with suspected sepsis were prospectively collected and tested using the developed method. Broad range 16S rDNA PCR and conventional culture analyses were performed in parallel. The pathogen DNA enrichment strategy resulted in an average removal of 99.99% of human DNA in 1ml blood samples.

One of the 8 samples was positive for Group A *Streptococcus* (*S. pyogenes*) by 16S PCR and the novel NGS based method. This sample was negative by culture, however a sample taken from the patient earlier that day was positive, also for Group A*Streptococcus*. The remaining samples were negative by culture. A total of 11 million Illumina reads were obtained from the enriched blood sample, of which 0.2% were Group A Streptococcus (the remainder were mainly human sequences). This was sufficient to provide 3x coverage of the *S. pyogenes* genome.

Conclusion: To our knowledge, this is the first description of culture independent non-targeted NGS based diagnosis of BSI. This study provides proof-of-concept that NGS based diagnosis of sepsis is feasible provided an efficient pathogen DNA enrichment strategy is used. With sufficient genome coverage, a comprehensive molecular antibiotic resistance profile of the pathogen can be generated.

Evaluation of QuantSeq 3' mRNA Sequencing with Spike-in Transcripts

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With the rapid development of NGS technologies, RNA-Seq has become the new standard for transcriptome analysis. Although the price per base has been substantially reduced, sample preparation, sequencing, and data processing are major cost factors in high-throughput screenings. Lexogen has developed the QuantSeq kit to reduce expenditures in these areas. QuantSeq provides an easy protocol to generate highly strand-specific NGS libraries close to the 3' end of polyadenylated RNAs within 4.5 h. The kit requires only 0.5–500 ng of total RNA input without the need for poly(A) enrichment or ribosomal RNA depletion since library generation is initiated by oligodT priming. First strand synthesis is followed by random-primed synthesis of the complementary strand, introducing Illumina- or IonTorrent-specific linker sequences. The resulting double-stranded cDNA is purified with magnetic beads, rendering the protocol compatible with automation.

To evaluate differential gene expression with QuantSeq, we used the FDA's Sequencing Quality Control (SEQC) standard samples A and B that are mixtures of reference RNAs with spike-in transcripts from the External RNA Controls Consortium (ERCC spike-in control mixes 1 or 2). The correct detection of differential gene expression (true-positive versus false-positive) as assessed with the ERCC dashboard (Munro et al., 2014) was higher for QuantSeq than for standard RNA-Seq. The numbers of ERCC transcripts detected was almost identical, even for read numbers down-sampled from 10 M to 0.625 M. QuantSeq reads almost exclusively map to the 3' end of annotated transcripts, and the thereby reduced level of required coverage reflects a 12-fold possible reduction in read depth when compared to standard RNA-Seq.

The QuantSeq protocol shows a superior strand specificity of >99.9 %, greatly facilitating the discovery and quantification of antisense transcripts and overlapping genes. The very high input-output Spearman correlation of 0.973 and 0.986 for ERCC mix 1 and mix 2 emphasizes QuantSeq's very high gene count accuracy. Computational demand and mapping time are also dramatically reduced, since no complicated coverage-based quantification is required. 6 QuantSeq data sets were aligned in only 35 min using the short read aligner Bowtie2 while the corresponding RNA-Seq data sets required 2 h 50 min using a splice-junction mapper, TopHat2.

QuantSeq is a robust and simple mRNA sequencing method. It increases precision in gene expression measurements as only one fragment per transcript is generated. At lower read depths, such focus on the 3' end results in higher stability of differential gene expression measurements. QuantSeq is ideal for increasing the degree of multiplexing in NGS gene expression experiments and is the method of choice for accurately determining gene expression at the lowest cost.

Munro et al. (2014) Nat. Commun. 5, 5125.

Next Generation Sequencing And Genetic Variant Analysis In Otoslerosis

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Otosclerosis is one of the main reasons of hearing loss (HL) in adult populations and very little is understood about etiology. Otosclerosis is caused by abnormal bone remodeling in the otic capsule. HL appears when one of the bones in middle ear (stapes) becomes stuck and the vibration and conduction of the sound through the ossicular chain is compromised. Otosclerosis can also affect the other ossicles (malleus and incus) and the otic capsule - the bone that surrounds the inner ear. Bone remodeling disorder of the ossicles leads to conductive hearing loss (CHL), disorder in the otic capsule to sensorineural hearing loss (SNHL) and when otosclerosis involves the small bones and the cochlea to mixed hearing loss. Disease normally appears in the third to fifth decade of life. The prevalence increases almost 7-fold with age and varies in different populations. At the moment, no cure is available, and the treatment comprises the use of hearing aid or replacement of effected stapes with a prosthesis (stapedectomy). Authors report that environmental and multiple genetic factors can cause development and progress of the otosclerosis.

The disease is very heterogeneous both clinically and genetically. It is usually inherited in an autosomal dominant pattern with variable penetrance. In development of otosclerosis, besides environmental factors, several genes may be involved, depending upon the individual, but it is not clear which genes or mutations in them contribute to the development of otosclerosis.

In recent years, next-generation sequencing technologies enabled detection of variants in a very large number of disease-associated genes, which were in most cases prohibitively expensive using previously available techniques. Here we present simultaneous screening for mutations associated with more than 700 diseases using the Ion AmpliSeq Inherited Disease Panel, focused predominantly on genes, which may be linked to development of otosclerosis.

In our study, we focused on one family with confirmed otosclerosis in some members. DNA was extracted from blood or saliva samples, and the ultra-high multiplex PCR was used to amplify the exons of 328 target genes. The prepared DNA libraries were sequenced on the Ion Torrent PGM sequencer. Both single-sample and trio analysis of variants was performed with the Ion Reporter cloud-based software.

The next-generation sequencing strategy and the trio analysis used in the present study proved to be a very quick and cost-effective approach to inherited disease genotyping, offering a level and depth of information that was unattainable with previously used methods.

Non-invasive Prenatal Testing (NIPT): Quantitation Of NGS Reads To Detect Fetal Trisomies

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Context: Cell-free fetal DNA (cffDNA) is detectable in maternal blood plasma starting from the 7th week of gestation and represents approximately 10% of the total cell free DNA (cfDNA) circulating in the blood. CffDNA can be utilized for the non-invasive prenatal detection of fetal trisomies 13, 18 and 21. This technique has been designated as non-invasive prenatal testing.

Objectives: Establishment of a highly sensitive NIPT workflow in a German genetics laboratory based on the quantitation of normalized next generation sequencing (NGS) reads of all chromosomes.

Methodology: A technology transfer project has been carried out in partnership with the US test provider Illumina/Verinata Inc. including a result analysis pipeline installed at the MVZ Martinsried (Prenatalis™-Test). After cfDNA isolation, genome wide NGS is performed with high

sequencing coverage (> 21 million reads per sample), followed by mapping reads to corresponding chromosomes. To correct for different chromosome-specific sequencing efficiencies, intra-run noise and inter-run sequencing variation, the read number of the chromosome of interest is normalized to the read count of a predefined reference chromosome set, which exhibits minimal sequencing variation. The resulting "normalized chromosome values" (NCVs) are compared to a training set of unaffected samples and tested for significance by Z-score statistics.

Results: Applying the described normalization method resulted in an extended dynamic range of NCV values compared to previously published quantitation strategies (Sehnert et al., 2011). Additionally, we obtained highly reproducible results from identical samples sequenced in another lab. The high sequencing coverage reduces the standard deviation of the chromosome specific read count and allows the correct classification of samples even with low amount of cell-free fetal DNA (so called low fetal fraction).

Conclusion: Accurate NGS-based NIPT for fetal trisomies requires an optimized strategy to correct for intra-run and inter-run sequencing variation. By using a set of "housekeeping" chromosomes for normalization, quantitation of cell-free DNA based sequencing reads can be performed in a highly reproducible manner indicating the presence or absence of a fetal trisomy 13, 18 or 21.

Literature: Sehnert, A.J., Rhees, B., Comstock, D., de Feo, E., Heilek, G., Burke, J., and Rava, R.P. (2011). Optimal Detection of Fetal Chromosomal Abnormalities by Massively Parallel DNA Sequencing of Cell-Free Fetal DNA from Maternal Blood. Clin. Chem. *57*, 1042–1049.

Optimization of Library Preparation Protocol for Next Generation Sequencing of Plant RNA Virus

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RNA viruses have small, simple genomes, which have a high level of diversity and present a special challenge for whole genome sequencing using traditional methods. The problems with virus whole genome sequencing can be avoided by utilizing next generation sequencing (NGS); a high-throughput sequencing methodology which generates millions of sequences simultaneously from one sample. The current NGS protocols are well optimized for large amounts of extracted nucleic acids. In total RNA preparations from infected plants, the amount of viral RNA is too low to be used in routine protocols.

The aim of our study was to sequence the whole RNA genomes of naturally occurring viruses in symptomatic cultivated and wild *Rubus species* using Ion Torrent technology.

The extracted total RNA was enriched for virus sequences by using the RiboMinus™ Plant Kit (Life Technologies). The kit is designed to selectively deplete ribosomal RNA from plant total RNA preparations. It removes plant nuclear, chloroplast and mitochondrial rRNA molecules from total RNA extracts.

After ribosomal RNA depletion cDNA library was prepared by Ion Total RNA-Seq Kit v2 library preparation kit (Life Technologies). The quality and quantity of prepared library was checked on Caliper LabChip GX instrument and sequenced with the Ion Torrent PGM instrument. Despite the initial design of the protocol aimed to enrich the sample for viral sequences, data analysis revealed a high percentage of plant sequences remaining in the sample (non-rRNA). A distinct peak was observed during quality control of cDNA library and it contained host related sequences. Therefore, we further optimized the library preparation protocol by adding an additional step of size selection during library preparation.

Quality control for SureSelect Strand-Specific RNA library preparation protocol using the Agilent 2200 TapeStation system

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Transcriptomics reveal global changes in gene expression that may contribute to the pathogenesis of a particular disease or help drive a fundamental biological process. RNA sequencing (RNA-Seq) has emerged as a promising and rapidly growing method for studying and characterizing cellular transcripts at single base pair resolution. Among different approaches for generating libraries for transcriptomics study, strand-specific library preparation method has added advantage over other methods. The Agilent SureSelect strand-specific RNA library preparation kit generates libraries with specific adaptors ligated to each strand enabling the identity of the DNA template strand of origin to be retained in downstream sequencing and data analysis. The high throughput deep-sequencing based RNA-Seq studies generate more data compared to RT-qPCR and array based methods yet they are expensive and time consuming study. Proper quality control (QC) steps within the library preparation process are therefore crucial to ensure successful sequencing. The Agilent 2200 TapeStation system offers an easy to use automated electrophoresis system with rapid analysis time as well as flexible sample throughput capabilities. Here, we compare the performance and capabilities of the RNA and D1000 ScreenTape assays for use on the Agilent 2200 TapeStation system with the respective assays run on the 2100 Bioanalyzer system. The results from the QC of the starting total RNA and final sequencing libraries generated from Agilent's SureSelect strand-specific RNA library preparation kit reveal that the RNA and D1000 ScreenTape assays are a match to the visual and quantitative results obtained with the RNA 6000 Nano and DNA 1000 Kits.

RNA Sequencing in degraded tumor RNA samples: Two natural enemies finally reconciled

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Carved in stone, painted on canvas or scratched in vinyl - expressions of human thoughts and feelings have been conserved over generations and been ever since an easily accessible treasure of knowledge and inspiration. When it comes to expression of genes conserved in FFPE or degraded RNA samples, things are different. Given the degradation of RNA due to fixation or other circumstances to shreds and pieces, the scientific treasure of potential insights and discoveries is everything but easy to unearth. Unfortunately, classic approaches often suffer from high sample amount input requirements and highly variable results. These hamper immensely the effective gene expression analysis of challenging FFPE or degraded but nevertheless precious and irreplaceable RNA samples.

In the current study strongly degraded total RNA samples from stomach endoscopies of patients with gastric carcinomas should be analyzed for gene expression changes between tumor regions and normal tissue regions. To overcome the described problems, we established a new RNA Sequencing Service workflow. The library preparation with the **Illumina TruSeq RNA Access** kit served as a key step, especially addressing low-quality RNA samples and requiring input amounts of as little as 20-100 ng. RNA was evaluated for usability by the newly established Illumina DV_{200} quality parameter. The subsequent stranded RNA Access library preparation included a sequence-specific enrichment technology. This enables reproducible transcript capture, not compromised by poly-A bias, using >425'000 probes, interrogating >210'000 targets of 21'415 genes of hg19 and thus covering 98.3% of RefSeq. By this approach, variability was diminished while required sequencing depth was greatly reduced. The IMGM´s FFPE RNA Sequencing Service was expanded by 2x75bp paired-end sequencing on the NextSeq500 platform (Illumina) and a comprehensive data quality control and analysis on the CLC Bio Genomic Workbench.

Sequencing resulted in a high amount of high quality paired end reads and a very high portion could be mapped to the human genome, regardless the level of degradation of the underlying RNA samples. The comparison of the resulting data with data from two of the samples, which underwent a classical mRNA library preparation protocol, showed that the used TruSeq RNA Access protocol enhanced the amount of mappable reads and diminished sequencing of intergenic regions. Thereby paired-end reads could be concentrated on meaningful gene regions in high sequencing depth, finally enhancing the focus for detection of significant differential expression levels and fusion genes.

Thus, the used approach of the IMGM´s FFPE RNA Sequencing Service powered by the Illumina TruSeq RNA Access kit serves as an efficient step towards gaining so far hidden transcriptomic insights in difficult to access RNA samples.

A method for assessing bacterial enrichment for the removal of human DNA for metagenomic analysis using qPCR and High throughput qPCR

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Chronic obstructive pulmonary disease (COPD) is characterised by irreversible obstruction of respiratory pathways and according to the World Health Organisation is the third most common cause of death worldwide. Despite being treatable and preventable, acute exacerbations contribute to high morbidity and mortality. Around 50% of respiratory exacerbations are associated with bacterial and viral infections, however the complexity of microbiome dynamics that determines the onset and course of exacerbations needs further exploration.

Deep sequencing of the bacterial 16S ribosomal RNA subunit gene has proved to be a powerful tool for human microbiome analysis as it specifically measures the bacterial community present in a sample, independent of the host DNA. However it fails to detect viruses, fungi and some bacteria. This approach also provides limited genetic information and cannot detect antibiotic resistance markers or differentiate strain types. Whole metagenomic sequencing of total DNA overcomes these drawbacks and, therefore, shows great potential for monitoring microbiome dynamics during and between exacerbations. However, whole genome sequencing also measures the human host DNA, which often represents the vast majority of the nucleic acid present in a patient sample. Consequently, clinical samples often require enrichment of bacterial DNA to reduce the human DNA content and thereby increase the ratio of bacterial vs. human DNA.

In this study, the NEBNext® Microbiome DNA Enrichment Kit (New England Biolabs) was assessed in terms of inter- and intra-sample repeatability for recovery of total and species specific bacterial DNA. Enrichment was performed on six replicates containing artificial human and bacterial DNA, and on 12 samples comprising extracted DNA from six COPD patients with or without spiked extraction control, composed of six bacterial causes of COPD exacerbation. Quantitative PCR of the human Alu repeat region and 16S rRNA assays was performed to determine changes in quantity of human and bacterial DNA, respectively. Additionally, species specific recovery was determined using high throughput qPCR (Biomark, Fluidigm) by quantitative analysis of 14 different pathogens.

NEBNext Microbiome DNA Enrichment Kit was shown to be an appropriate tool for removal of human DNA from artificial and clinical samples. It increases the ratio of bacterial vs. human DNA while reproducibly retaining the ratios between different pathogens, thus improving whole genome metagenomic sequencing in clinical samples. Additionally, qPCR for Alu and 16S rRNA genes, and high throughput qPCR for species specific quantification proved to be a valuable method for evaluating a bacterial DNA enrichment method.

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Non-coding RNAs (Poster 17 - 25)

A complete workflow for high-throughput isolation of serum microRNAs and downstream analysis using qPCR: application to cancer biomarker discovery

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Biomarkers are invaluable tools for cancer detection, diagnosis, patient prognosis and treatment selection. An ideal biomarker should be easily assayed with minimally invasive medical procedures but possess high sensitivity and specificity. Although many candidate biomarkers for various diseases have been proposed in the literature, very few have made their way to clinical use, and for certain types of cancer there are no reliable (biomarker) options.

For instance, it is well recognized that prostate cancer is over-diagnosed and over-treated. Approximately 230,000 men were diagnosed with prostate cancer in 2014, however, almost four times as many had a biopsy performed after routine screening (PSA test). The identification of biomarkers that can assist with risk-stratification of patients would be highly beneficial, as their use could potentially decrease the complications and morbidity related to unnecessary biopsy and prostatectomy.

The circulating microRNA (miRNA) in blood could potentially fill this void, providing biomarkers suitable for real-time detection and application to the management of prostate cancer patients. The main advantages of miRNAs versus longer molecules (mRNA, lncRNA) include: (i) smaller number of sequences <2000 and thus ease of analysis (ii) stability due to small size ~16-27 nt and thus robustness of detection.

The objective of this project was to develop a set of reagents and tools optimized for efficient recovery and analysis of circulating miRNAs. Over the last two years, our team built and optimized a complete workflow for (1) high throughput isolation of total serum RNA using a novel MagMax mirVana RNA isolation kit and KingFisher Flex Duo automated system and (2) downstream analysis of microRNA profiles by qPCR using next generation TaqMan assays and cDNA synthesis kit.

The effectiveness of this workflow is exemplified by analysis of a panel of miRNAs in samples derived from the serum of patients with metastatic prostate cancer versus healthy donor controls. Several promising microRNA biomarkers were identified, discriminating the two groups. Following this fast and easy workflow suitable for serum and other body fluids, disease-specific microRNA signatures can be identified and used as biomarkers.

A High Performance qPCR Assay Platform for Circulating MicroRNA Biomarker Development

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Recent studies showed microRNAs are released from tissues and cells into circulation and their expressions correlate with specific disease conditions. These microRNAs are protein bound or encapsulated in vesicles and remain stable in biofluids. Such attributes made circulating microRNAs promising non-invasive candidate biomarkers for disease early detection, prognosis and treatment selection. However, significant technological challenges remain, contributing to data inconsistencies across studies and hampering the development of accurate and robust miRNA based tests. Efficient identification of clinically relevant miRNA biomarker requires a well-designed study and an integrated workflow with consistent sample extraction, robust RT-qPCR quantification, and stringent statistical analysis. In addition, control measures are essential to monitor and normalize technical and biological variations that confound the interpretation of data. We have developed an enabling platform based on proprietarily designed and validated qPCR assays that detect extremely low levels of circulating microRNAs with high sensitivity and specificity. This platform routinely detects over 400 microRNAs from 200 microliters of serum and plasma with a dynamic range span over eight orders of magnitude and total workflow variation of less than 0.5 Ct. The platform has been independently validated by accredited molecular diagnostic laboratories and pharmaceutical partners, showing superior performance to existing commercial assays. Using this platform, multi-miRNA biomarker panels have been identified and validated in cross-population cohorts for early stage breast cancer (n=600, AUC>0.93) and gastric cancer (n=800, AUC>0.9). In addition, a series of other circulating miRNA signatures have been identified and being further developed for potential applications in early detection of ovarian cancer, stratification of patients with cardiovascular diseases as well as drug response predictions.

Serum MicroRNA Biomarker Panels for Non-invasive Detection of Gastric Cancer

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Gastric cancer is the 4th most common cancer (>1 million cases per year) and the 2nd most common cause of cancer deaths worldwide. Majority of gastric cancer are diagnosed at an advanced state of the disease due to the lack of a cost-effective method for cancer detection. Currently, endoscopy is the only reliable method for early diagnosis but it is limited as a screening test due to the high cost and risk. A less invasive screening test for gastric cancer is highly desirable. To develop serum microRNA based signature for gastric cancer detection, more than 500 miRNAs were quantified by MiRXES® qPCR technology in the sera of 238 gastric cancer subjects and 238 age, gender and race matched gastric cancer free healthy subjects, serving as the discovery set. From such measurements, 191 miRNAs were reliably detected in all the serum samples where 75 informative ones were identified to be significantly (false discovered corrected P value lower than 0.01) altered between gastric cancer and normal subjects. Multivariate miRNA biomarker panels were then formulated by sequence forward floating search and support vector machine using all the quantitative data obtained for the expression of 191 miRNAs. Multiple times of two fold cross-validation were performed *in silico* where the biomarker panels using at least 8 miRNA consistently produced values of ≥0.87 when represented as areas under the curve (AUC) in the receiver operating characteristic (ROC) curve. Several models including 12-24 microRNAs were further optimized based on the discovery set and validated in two blinded studies with fixed algorithm and threshold definition: Korea population (n=183, 74 cancer cases) and Chinese population (n=89, 20 cancer cases). The prediction algorithms had sensitivities of around 90% with 80% - 85% specificities in both validations. The scores generated from the linear model had AUC of >0.90 for the Korea population validation and >0.85 for the Chinese population validation. These signatures are currently being further verified in additional independent cohorts from multiple countries and a retrospective clinical study is being carried out in Singapore.

Microparticles Associated MiRNA Release By M2 But Not By M1 Macrophages From Patients With Psychiatric Diseases.

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There is profound evidence that psychiatric disorders are associated or even causally related to sterile or infection associated inflammation. Generally, are associated with infections and tissue trauma. Physiologically, the M1 phenotype of inflammatory macrophages differentiates into M2 macrophages which are anti-inflammatory and condition the resolution of inflammation by profound immune suppression. In diseases with chronic inflammation, M2 macrophages are either not activated and a sterile inflammation persists, or the inflammatory immune response fails to induce a protective immune response. M1 and M2 macrophages differ by a number of phenotypic markers as well as by their cytokine secretion pattern. They are further distinct by the expression of the P2X7 ion channel which can be stimulated by extracellular ATP related to tissue trauma. P2X7 activation leads to a calcium influx which activates the inflammasome to process IL-1β if the intracellular pro-IL1 levels are significant. In addition, P2X7 activation leads to the release of microparticles which may reprogram other target cells following membrane fusion. Using patch clamping, we determined ATP-stimulated P2X7 ion flux and simultaneously determined the changes of plasma membrane surface area - correlating with microparticle release - by resistance measurements. Microparticles released were enriched by ultracentrifugation and RNA preparations were performed and subjected to miRNA quantification using after cDNA synthesis with the appropriate kit from Quanta Bioscience. The expression in miRNAs like let7a, mir-16, mir-21, mir-93, mir-132, mir-146A, mir-204 and mir-210 was analysed with specific forward primers and Quanta´s universal reverse using PerfeCta SYBR Green SuperMix on a LightCycler480. Expression patterns were shifted depending on the immune-priming. The joint interpretation of protein expression, microparticle characteristics and changes within certain miRNAs seems to be a valid tool to illuminate the role of the immune system in psychiatric diseases.

Development Of Non-invasive MicroRNAs Tests As Tools For Early Detection Of Colorectal Cancer

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The incidence of colorectal cancer (CRC) has increased significantly as to become the leading cancer in Taiwan in recent years. The 5-year-survival rate of colorectal cancer can be as high as 90% when diagnosed at the initial stage. Hence, early detection and immediate surgery removal of cancerous lesion are crucial for disease cure. However, the diagnostic accuracy of the conventional blood carcinoembryonic antigen (CEA) or immune-fecal occult blood test (iFOBT) for early detection is limited due to the insufficient sensitivity or specificity, respectively. In order to improve the detection rate of CRC, we established a CRC-related microRNA (miRNA) panel containing 46 miRNAs selected from the literatures in past ten years. We then analyzed those miRNAs in stool and plasma samples as non-invasive screening tests by TaqMan based reverse transcriptase real-time PCR methods. To evaluate if those miRNA levels in stool and plasma can be truly reflected the local tumor biology, we compared those miRNA levels in paired tumor and adjacent normal samples from 62 CRC patients, as well as in stool and plasma

samples of those patients. We alsodetermined those miRNAs in stool and plasma samples from age- and gender-matched normal controls. Among the 46 microRNAs, 28 microRNAs were consistently expressed and reliably detectable in all 3 types of clinical samples, and also showed a positive correlation between tissue and stool samples (R=0.73), tissue and plasma (R=0.72), and plasma and stool (R=0.88). At least 5 microRNAs were found highly expressed in all 3 types of sample. This is the first study linked the microRNA expression in tissue, stool and plasma samples from individual CRC patients, supporting the potential application of candidate microRNAs in stool and plasma samples for early detection of CRC.

microRNA assessment in cerebrospinal fluid for diagnosis of primary central nervous system lymphoma

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Introduction: Precise differential diagnosis of primary central nervous system lymphoma (PCNSL) and non-neoplastic diseases of the central nervous system (CNS) is a prerequisite for proper treatment. Diagnosis of PCNSL is still challenging, despite the use of brain imaging techniques, histopathological examination of brain biopsies, cytological and flow cytometric examination of cerebrospinal fluid (CSF). Futhermore, brain biopsy carries risk of neurological complications, and sometimes is unfeasible due to inaccessible location of the lesion.

Aim: This study was aimed to optimize the procedure for measurement of microRNA expression levels in CSF and to evaluate the utility of miR-21, miR-19b-1 and miR-92a in CSF for the differential diagnosis of PCNSL vs. non-neoplastic diseases of the CNS.

Material: Leftover CSF samples were collected for routine diagnostic purposes from patients consulted at the Cancer Centre and Institute of Oncology in Warsaw, suspected of the PCNSL.

Methods: Measurements of the expression levels of miR-21, miR-19b-1, and miR-92a were performed by RT-qPCR.

Results and Conclusions: In this study a procedure for CSF sample preparation has been developed for reproducible measurement of microRNA expression levels. The preliminary data showed distinct expression profile of miR-21, miR-19b-1 and miR-92a in CSF from patients with PCNSL compared to non-neoplastic diseases of the CNS, suggesting the potential utility of the microRNAs for the differential diagnosis of PCNSL.

How to be one-step ahead by streamlining an existing microRNA cDNA synthesis kit

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As microRNA is not polyadenylated by nature, classical reverse transcription is unfeasible for microRNA cDNA generation due to its sheer shortness. One possible option to bypass this fact is the artificial polyadenylation of microRNAs prior to a reverse transcription with oligo-dT adaptor as primers. We tested kits from various vendors and found that Quanta's qScript miRNA cDNA synthesis kit met best our needs. The only unpleasant thing about it was the necessity of stepwise pipetting of buffers and enzymes with opening the tube during the process. Not only does this procedure increase the total hands-on time, but also chance of contamination. Here we present the adjustments to protocol and components to make this kit more feasible in handling while keeping its standard performance.

Long Non-Coding RNAs Implicated in the Pathogenesis of Parkinson Disorder

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It is well-known that only a small part of the human genome (appr. 1-2%) encodes proteins, however, more than half of our genome is transcibed to RNA molecules. The function of these non-coding RNAs is subject of extensive investigations: in the recent years, novel RNA classes has been described, one of these are the so called long non-coding RNAs (lncRNAs). These molecules are defined as transcipts longer than 200 nucleotides without an extended open reading frame (ORF). Their maturation processes are very similar to that of messenger RNAs, however, compared to mRNAs, they possess a higher cell- and tissue specifity.

LncRNAs are particularly abundant in the central nervous system and due to their strict spatial-temporal expression, they might play key role in brain development. The role of lncRNAs is also prominent in the adult brain: an emerging number of publications support that their disfunction promote neurodegenerative disorders such as Parkinson-, Alzheimer- and Huntington-disorder. Our aim is to investigate whether single nucleotide polymorphisms (SNPs) of genes encoding lncRNAs contribute to the development of Parkinson disorder.

For that we performed an *in silico* analysis using a publically available RNA-Seq database (http://www.broadinstitute.org/genome_bio/human_lincrnas/) and selected genes that are highly expressed in the brain tissue. In addition, six lncRNAs were chosen based on literature data, which have been previously proven to be involved in neurodegenerative disorders: HAR1F, Uchl1-AS, PINK1-AS, Sox2-OT, BC200 and ANRIL. With the help of the ENSEMBLE Database, we are searching for the non-coding exonic variants of these transcripts, possessing a Global Minor Allel Frequency (MAF) higher than 0.1.

As a pilot study, we aim to examine the genotype distribution and allele frequency of 15 selected polymorphisms in healthy volunteers and patients with Parkinson disorder, to show whether these SNPs are associated with the development of the disease.

Exosomal microRNAs in urine - promising biomarkers for disease

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microRNAs 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 human protein coding genes are regulated by this small class of RNA (~2000 miRNA).

The study of extracellular microRNAs and their potential as pathophysiological markers has greatly expanded in the last couple of years. The high relative stability of microRNAs 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 in cancer.

We have applied Exiqons highly sensitive LNA™-based qPCR platform for detection of microRNAs, which has enabled microRNA profiling in biofluids where levels are extremely low. The platform uses a single RT reaction to conduct full miRNome profiling and allows high-throughput profiling of microRNAs without the need for pre-amplification. Thousands of biofluid samples including serum/plasma and urine have been profiled to determine normal reference ranges for circulating microRNAs as well as to identify biomarkers of disease. Extensive data qualification and analysis methods have been developed and these are central in securing high quality data from biofluids. The methods can quickly and robustly be applied in biomarker discovery and validation projects. We will present data showing detection and stability of microRNAs in urine, and examples of differential urinal expression from our collaborative cancer diagnostic projects. Also we recently developed a new exosome enrichment method and will present data showing a characterization of the exosome fraction obtained with this method and how the method enhances detection of microRNAs from urine. We will also present a comparison of microRNA profiles obtained with this method to profilesobtained with different commercially available exosome isolation methods.

Biomarker Discovery (Poster 26 - 41)

Applicability of Blood RNA Quality Biomarkers for EDTA Tubes in High-Throughput qPCR Gene Expression Experiments Using BioMark System from Fluidigm

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It is increasingly recognized that pre-analytical factors, if not properly identified and controlled, can have an effect on sample quality and, consequently, on the quality of molecular analysis. A simple factor as a selection of the type of container for sample processing can make an important difference in the obtained results and can have a significant impact on the stability and levels of mRNA measured in biomarker studies. It has been described previously that dysregulation of some genes in K_2EDTA collection tubes can occur because of the absence of any stabilizer of gene expression in the collection tube [1,2]. So, preferably and if possible, blood should be collected to special collection tubes, for example PAXgene Blood RNA Tubes, that offer the immediate stabilization of blood RNA [1]. However since recently, there is a solution for controlling the stability of samples already collected to frequently used K_2EDTA collection tubes, which is a panel of quality RNA biomarkers validated within SPIDIA consortium [3,4]. Here, we propose one possibility how to apply Blood RNA quality biomarkers for EDTA tubes using high-throughput qPCR instrument BioMark from Fluidigm and multivariate analysis tools as principal component analysis and Kohonen's self-organizing map.

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A Reliable Method to Quantify Transcript Isoform Expression of Apoptotic Genes Involved in the Response to Tyrosine Kinase Inhibitor (TKI) Therapy in Chronic Myeloid Leukemia (CML)

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CML is caused by a chromosomal abnormality (Philadelphiachromosome) resulting in the BCR-ABL1 fusion gene and constitutive tyrosine kinase activity. BCR-ABL1 TKIs have revolutionised therapy for CML patients, however, patient responses are heterogeneous, and accurate prognostic biomarkers at diagnosis are lacking. TKIs kill CML cells by upregulating pro-apoptotic BCL2 proteins, such as BIM, BAD and BMF, in the intrinsic apoptotic pathway. Recent data suggest that inter-patient variation in pre-treatment BIM expression may modify the apoptotic response to TKIs in solid tumors, impacting clinical outcome. Our goal is to identify prognostic biomarkers, at the time of CML diagnosis, by examining the relationship between the expression of BCL2 family isoforms and treatment response.

We developed a Fluidigm Dynamic Array assay to simultaneously measure the expression of 33 transcript isoforms of 10 BCL2 genes. Careful primer design enabled specific isoform amplification, despite the highly homologous sequences, which was confirmed by gel electrophoresis and sequencing. Literature searches, the RefGenes database and geNorm analysis were used to select appropriate genes for data normalization. Custom modification to the standard pre-amplification PCR primer pool enabled accurate isoform quantification. Assays were validated by comparing Ct data to conventional RT-qPCR data. Analysis of intra- and inter-assay variation demonstrated high assay reproducibility. The ΔΔCt relative quantification method was used to calculate fold changes in expression. Several HTqPCR package tools and 'in-house' R scripts were used for data analysis, graphics and statistical analysis.

Four CML cell lines, known to upregulate pro-apoptotic BIM and BMF isoforms in response to TKI treatment were chosen for a pilot study. When comparing pre- and post- TKI treatment samples, we detected expression increases of up to 50- and 100-fold in pro-apoptotic BIM and BMF isoforms, respectively. This was accompanied by increased caspase-3/7 activity, confirming intrinsic pathway activation.

In conclusion, we have developed a high throughput RT-qPCR assay to quantify isoform expression of BCL2 family genes involved in the apoptotic response to TKI therapy. Our pilot study demonstrated that we could reliably detect relative isoform changes in response to TKI treatment, as expected. To our knowledge, we are the first to use Dynamic Arrays to measure highly homologous sequences, which required modification of the pre-amplification protocol to enable accurate isoform quantification. We plan to study isoform expression in >600 CML

diagnostic samples. This data will be correlated with known clinical outcomes, with the aim to develop a new biomarker based prognostic risk score.

Biomarkers for Metastatic Colorectal Cancer

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In patients who have been diagnosed with a cancer, biomarkers have many potential applications including differential diagnosis, prediction of response to treatment and monitoring of progression of disease. To identify potential biomarkers, large scale gene expression profiling has become an essential tool for the biological and medical investigations of pathological samples. However, this approach is often limited by the availability of large amounts of biological sample for RNA extraction.

Our approach is based upon the concept of using microfluidic droplets, to act as distinct miniature reactors, from which we will be able to quantify gene expression levels of both normal and cancer samples taken from a cohort of patients. We are focusing on genes that are regulating the extracellular matrix (ECM) environment. The ECM is a central component of the tumour microenvironment, housing several other cells including inflammatory cells and active fibroblasts, while feeding biological information to the cells to control cell differentiation and proliferation. We are developing a gene expression profile focusing on stroma related genes in colorectal samples. The need for higher throughput with increased level of specificity coupled with reduced volumes of reagents has become a dominant factor for biomarker discovery. Microfluidics provides numerous advantages such as economies of scale, parallelisation, automation, increased sensitivity and precision that come from utilising small volume reactions.

In a small patient cohort of matched benign, stage I, stage II, stage III and documented metastatic samples, we have demonstrated a pattern of ECM genes that are differentially expressed between normal and diseased tissue of the same patient. Integrin & matrix metalloproteinase (MMP) families are among those most dysregulated in 24 patient samples analysed. When expanded, this profile may help separate colorectal cancer into distinct 'extracellular matrix protein expressing' groups from which we will be able to develop robust diagnostic and prognostic tools that can be applied in a clinical setting.

Characterization of Antigenic Profiles of Primary Non-small Cell Lung Cancer Tumors and Lung Cancer Cell Lines for Vaccine Development

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Cancer cell lines might serve as a universal source of tumor antigens in the development of cancer vaccines. Immunogenic high hydrostatic pressure-killed cancer cell lines are used for dendritic cell-based active cellular immunotherapy of prostate and ovarian cancer. We investigated here whether commercially available non-small cell lung cancer (NSCLC) cell lines overlap in selected tumor-specific or tumor-associated antigens with primary NSCLC tumors (n=30).

A panel of 26 antigens was tested by RT-PCR in selected lung cancer adenocarcinoma cell lines A549, H596 and H226, squamous cell carcinoma cell lines H520, SK-MES-1 and H226 and large cell carcinoma cell lines H460 and H661. We found that out of 26 antigens, 17 antigens were differentially expressed in more than one lung cancer cell line. From these, 10 antigens (MAGE-A3, Prame, 5T4, Her2/*neu*, EpCam, MUC1, STEAP, SOX2, Survivin and WT1) were expressed by at least 4 lung cancer cell lines. The expression of these antigens was significantly enhanced in primary NSCLC cancer tumors in comparison to control non-tumoral lung tissue and peripheral blood mononuclear cells. In addition, their expression was confirmed on a protein level by immunoblotting and flow cytometry.

This data showed that selected antigenic profile of lung cancer cell lines overlapped with antigenic profile of primary NSCLC tumors which suggest that lung cancer cell lines would be suitable for generation of dendritic cell-based vaccine for immunotherapy of NSCLC.

Development of an in-house method to quantify BCR-ABL1 p210 transcripts aligned to the International Scale standards with high sensibility

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The BCR-ABL1 fusion gene is a result of a translocation involving chromosomes 9 and 22, and is found in nearly all patients with chronic myeloid leukemia (CML). Depending on breakpoint, BCR-ABL1 can encode different proteins. The most common is p210 (with 210 kDa), an aberrant tyrosine kinase known to be critical for the disease; its activity is inhibited by Imatinib, a selective targeted designed drug, the first clinically available to treat CML. Molecular monitoring during treatment is based on quantitative RT-PCR (qRT-PCR), targeting BCR-ABL1 transcripts in blood or bone marrow samples. Since lots of different extraction and qRT-PCR methods, equipment and reference genes are used for this quantification, big variation is observed and difficult comparison among laboratory results, confuses patients and physicians, and hampers therapeutic conclusions. So, efforts have been made towards standardization of methodology, with the adoption of an International Scale (IS) for reporting BCR-ABL1 results. We developed an in-house method which quantifies the b2a2 and b3a2 p210 transcripts and the ABL1 gene as reference. The RNA extraction procedure was established using 10mL of peripheral blood or 5mL of bone marrow using a customized TRIzol® protocol which yield around 35-40ug of RNA with great quality a Bioanalyzer´s RIN around 8.0. Starting from 3ug of RNA we perform the cDNA synthesis using the High Capacity kit. Our qPCR assay shares the same forward primer and TaqMan-MGB probe for both b2a2 and b3a2 and has specific reverse primers for each transcript in a multiplex reaction. In a separate reaction, the reference gene ABL1 is amplified. We also validated the use of ipsogen Standard Curves. Our qRT-PCR assay is able to detect (LoD) as little as 10 copies for p210 transcripts and for ABL1 and to quantify (LoQ) 20 copies each. In order to follow the international guidelines and avoid false negative results, our results are considered trustful when, at least, 10.000 copies of ABL1 are quantified. To align our results to the International Scale standard, we validated the use of Asuragen ARQ IS Calibrators. Our measurements of the provided reference material showed a correlation (measured by the R^2 between our results and the expected results) of 98% with minimal bias. Regarding to the test sensibility, we are able to access the quantification of more than 1 million of ABL1 copies, which means, for p210 undetectable samples, a major molecular response with 6 logs of reduction (MMR6.0).

Diabetes Causal Molecular and Cellular Mechanisms underlying the *COBLL1* locus

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Genome-wide association studies repeatedly associated numerous variants at the *COBLL1* locus (2q24.3) with phenotypes relevant to lipid metabolism and glucose tolerance. To establish the causal regulatory variant driving these signals, we utilize ENCODE-/ Roadmap-chromatin state maps and the comparative regulatory motif module analysis PMCA. We find a likely causal variant with remarkably specific enhancer marks in adipose-derived mesenchymal cells and an enrichment for cross-species conserved motif modules. Reporter gene and electrophoretic mobility shift assays reveal adipocyte specific allelic differences in transcriptional activity and a differential transcription factor-DNA complex (risk > non-risk allele). Conditional on the identified transcription factor, we further show an allele specific expressional regulation of the downstream target *COBLL1*. Expression correlation analysis in primary human adipocytes and shRNA-mediated *COBLL1* silencing in human SGBS adipocytes, reveal marker gene expression and cellular phenotypes consistent with perturbed fat metabolism and type 2 diabetes. We here provide evidence for a functional regulatory role of an intronic *COBLL1* variant, specifically in human adipocytes, further elucidating the genotype-phenotype association at the *COBLL1*locus.

Evaluation of Bias Associated with High Multiplex Target-Specific Pre-amplification

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We developed a novel PCR-based pre-amplification (PreAmp) technology that can increase the abundance of over 350 target genes one million-fold. To assess bias introduced by PreAmp we utilized ERCC RNA reference standards, a model system that quantifies measurement error in RNA analysis workflows. We assessed three types of bias: amplification bias, dynamic range bias and fold-change bias. We show that PreAmp does not introduce significant amplification and fold-change bias, even under high multiplex and high amplification conditions. We do detect dynamic range bias if a target gene is highly abundant and PreAmp occurred for 16 or more PCR cycles; however, this type of bias is easily corrected. To validate PreAmp performance in a gene expression profiling experiment, we analyzed a panel of genes that are regulated during differentiation using the NTera2 stem cell model system. We find that results generated using PreAmp are statistically equivalent to results obtained using standard qPCR without the pre-amplification step. Importantly, PreAmp maintains patterns of gene expression changes across samples; the same biological insights would be derived from a PreAmp experiment and a standard gene expression profiling experiment. Our PreAmp technology can thus facilitate accurate analysis of extremely limited samples in gene expression profiling experiments.

Gene Expression Profiling of Circulating Tumor Cells and Peripheral Blood Mononuclear Cells of Breast Cancer Patients

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Despite the advantages of diagnosis and treatment, breast cancer is one of the most common malignant cancer diseases. Despite the fact that mortality of this disease has been decreasing in the last years nearly one-third of patients eventually die due to formation of distant metastases. Circulating tumor cells (CTCs) play a key role in metastatic spread of primary tumors, therefore molecular characteristic of CTCs may improve the prediction of metastases formation and better treatment decisions. We hypothesized, that CTCs gene signature could be also detected in peripheral blood mononuclear cells (PBMCs) of patients.

We performed gene expression profiling of CTCs as well as PBMCs in the group of breast cancer patients (*n = 147*) using the gene panel covering 55 breast cancer associated genes. Gene expression profiling showed 19 genes with significantly different expression in CTCs compare to control group. 7 genes were significantly up-regulated in CTCs and 5 genes (*EpCAM, IGFR1, UPA, VEGFA* and*VEGFR1*) were expressed exclusively in CTCs. This data showed that CTCs of breast cancer patients mainly overexpress genes involved in proteolytic degradation of extracellular matrix as well as genes playing important role in EMT process of cancer cells. All findings lead to urokinase plasminogen system which was preferentially activated in CTCs

Gene expression profiling of PBMCs of breast cancer patients revealed 17 genes with significantly different expression between the groups. We also showed that specific gene expression profile of PBMCs of breast cancer patients may have a prognostic value. We identified genes that are associated with tumor grade and formation of metastases.

Systems Biology Using Single Cells

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Systems biology aims to decipher the molecular interactions that underlie biological responses by collecting a multitude of measurements on cellular attributes. Examination of the genomic landscape coupled to RNA expression signatures facilitates a high power of analysis for illuminating frameworks of modular circuitry and gene regulatory networks. A short-coming of many systems biology study designs has been the query of large numbers of pooled cells with the resultant global population averaging of interrogated parameters. Individual and distinct cells within large populations often instigate significant biological cascades; however, their contributions to measurements are masked. Accessing system-level datasets on such important subpopulations requires single-cell analytical approaches. Recent advances in integrated fluidic circuitry have enabled the development of new technology for single-cell analysis: the Fluidigm® C_1^{TM} Single-Cell Auto Prep System. The C_1 System readily integrates single-cell isolation and generation of templates for both qPCR and next-generation sequencing analysis. We illustrate systems biology profiling for fundamental biomarkers using cancer and stem cell lines. These results highlight the individuality of cellular states in seemingly homogeneous cell populations. Taken with newly emerging applications and workflows, the C_1 System is becoming a powerful addition in the array of technologies to further systems biology investigations.

A Micro-fabricated Single Cell Isolation System To Process 1000s Of Individual Cells For Genomics Applications

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Understanding biological heterogeneity at a single-cell level requires new technologies that miniaturize and automate the isolation of thousands of individual cells and process these individual cells for applications that use NGS or qPCR. Microfluidic technologies have streamlined single cell genomics applications but can do at best 96 individual cells at a time and costs over $20/cell. Droplet-based technologies have been shown to process thousands of cells but also hold several productizing challenges including handling emulsion oils and optimizing reaction efficiencies in picoliter volumes. In its first product version, WaferGen aims to increase throughput ~50 fold while simultaneously decrease cost at least 10-fold by engineering a simple device based on its proprietary, scalable, micro-fabricated chip, SmartChip, which contains an array of 5184 individual polymer-coated nanoliter wells drilled into a metal alloy substrate. The SmartChip platform has been used successfully for qPCR, genotyping and targeted sequencing applications and is proven for its flexibility in processing multiple samples in a single chip using the MultiSample NanoDispenser (MSND), a microsolenoid controlled reagent dispenser that can dispense volumes as low as 40 nl into the SmartChip wells. We will present data to support the reproducible dispensing of individual cells at a >80% occupancy rate (~4000 cells) and barcoding individual cells with unique molecular identifiers by adapting SCRB-seq, a Single Cell RNA Barcoding and Sequencing strategy to measure 3' differential gene expression

Upstream Open Reading Frames Regulate Cannabinoid Receptor 1 Expression Under Baseline Conditions And During Cellular Stress

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The endocannabinoid system (ECS) plays a crucial role in the regulation of a variety of physiological functions, such as learning and memory processing, vegetative control, energy homeostasis, immunity and stress response. It acts through different endogenous endocannabinoids which are able to bind to the cannabinoid receptor subtypes 1 and 2 (*CNR1* and *CNR2*). The *CNR1* is not only associated with phenotypes such as cognitive performance, addiction and anxiety, but is also known to be crucially involved in cellular responses to acute and chronic stress conditions. The molecular mechanisms leading to altered *CNR1* expression under acute or chronic stress exposure are not completely understood so far. It is known that the 5'- and 3'untranslated regions (UTRs) of genes can harbor regulatory elements, such as upstream open reading frames (uORFs) that are capable of influencing the expression pattern of the main protein coding region.

In our study, we investigated the influence of putatively functional uORFs present in the untranslated regions of the five mRNA variants of the human *CNR1* gene on transcription and translation under baseline conditions and various stress conditions *in vitro*. The functional analysis performed with reporter gene assay and quantitative realtime PCR revealed that two of these variants contain upstream open reading frames that modulate gene expression both under baseline condition and conditions of cellular stress. Thus our findings suggest that the functionally relevant uORFs found in the 5'UTR variants of *CNR1* are part of the cellular stress response mechanisms.

Virus Serotyping by TaqMan and SYTO 9 in Single Reaction

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The aim of this study was to develop a methodology based on the reverse transcription - quantitative PCR - able to distinguish between particular serotypes of dengue virus, a highly pathogenic virus transmitted by mosquitoes. The advantages of RT-qPCR based methods are suitability for early diagnostics or higher sensitivity and specificity compared to serological tests. Molecular techniques can also provide additional information, e.g. sequencing data.

Based on the *in silico* study comprising 120 sequences of all kinds of serotypes, the conserved sites of the genome could be predicted. The region of 5' UTR and capsid gene was chosen as a target for primers. Primers were designed to be universal for all of four dengue serotypes and so, a degenerated base is presented in each of primers. SYTO 9, the third generation intercalating dye with greater saturation of DNA, was used for melting analysis. Distinct melting temperatures between serotype 1 and 2 are suitable for determination of these serotypes. TaqMan probes were designed for serotype 3 and 4 taking the advantage of deletion/insertion between these serotypes, respectively.

Because the genome of dengue virus is formed by RNA, a treatment with DNase I prior to reverse transcription was carried out to overcome false-positive results. Moreover, the specificity of the assay was verified by using samples of selected viruses, bacteria or human DNA. Further, the robustness of the assay was examined on the set of simulated field samples such as dust, washing detergent, chalk etc. A few clinical samples were possible to analyse and determine the right serotype. The whole assay was developed according to MIQE guidelines with proper efficiency, as well as intra- and inter-assay variation and limit of detection assessment.

The scientific work presented here demonstrates a quick detection system combining TaqMan probes and melting temperature analysis as a differentiation tool for particular dengue virus serotypes in a single reaction.

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ZFP64 – a predictive RNA biomarker for cancer treatment with the HDAC-inhibitor Resminostat

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Introduction: IMGM offers a plethora of genomic services for identification and validation of RNA/DNA biomarkers in clinical and research studies. Together with 4SC, a set of RNA biomarkers was established to be evaluated in clinical trials as pharmacodynamic markers for the treatment with Resminostat, a HDAC inhibitor currently in phase II clinical development by 4SC. The analysis of gene expression data from two trials, namely the SHELTER trial (hepatocellular carcinoma HCC patients) and the SAPHIRE trial (Hodgkin's lymphoma HL patients) showed that the expression level of one of the marker genes at baseline before treatment start significantly correlated with median overall survival.

Materials and Methods: The differential gene expression of human PBMCs treated ex vivo with several HDAC inhibitors was analyzed using microarrays. Subsequently, gene regulation in response to Resminostat was validated by further microarray and qPCR analyses. Finally, 10 genes showing constant and robust expression changes were selected as biomarker panel for Resminostat activity in clinical trials.

The SHELTER study included patients with advanced stage HCC and radiological progression under 1st line Sorafenib treatment. The patients received either Resminostat or a combination of Resminostat and Sorafenib. The SAPHIRE study included patients with relapsed or refractory HL and consisted of a treatment with two dose groups of Resminostat only.

Results and Discussion: Resminostat in combination with Sorafenib increased the median overall survival of HCC patients to 8.1 months (SHELTER), whereas the SAPHIRE study showed a disease control rate of 54%. Gene expression analysis of blood samples from patients under Resminostat treatment verified the pharmacodynamic potential of the RNA biomarker panel including ZFP64 in the clinic. Exploratory analysis of the clinical data revealed, that a high ZFP64 mRNA expression at baseline in blood samples of patients was indicative of extending median overall survival compared to low ZFP64 mRNA baseline expression.

Conclusion: In vitro screening of cells treated with Resminostat revealed a robust pharmacodynamic RNA biomarker panel. In a clinical setting, high baseline expression of ZFP64 mRNA was shown to correlate with an increase in median overall survival under Resminostat treatment. Therefore, ZFP64 will be further evaluated in controlled clinical studies for its potential as a predictor of Resminostat responsiveness in cancer patients, opening the path to a personalized cancer treatment with Resminostat.

Sensitive, specific and highly-multiplexed qPCR using MNAzymes and PASS primers

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qPCR has revolutionised molecular diagnostics and rapid advances in NGS are providing new breakthroughs in the field. When comparing approaches, it is apparent that each provide advantages for specific applications but neither provides a “one size fit all”. For example, sequencing generates large amounts of data and can provide the broadest view of complex diseases such as cancer where mutation status may be informative for efficiency or resistance to therapy. However, it can take days to weeks to complete and, since it lacks the ability to detect minor alleles present at < 1%, it may not be ideal for applications such as monitoring cancer patients by liquid biopsies. In contrast, qPCR is exquisitely sensitive and ideal for this application. Techniques such as ARMS allow detection of point mutations in clones present at only 0.1%, but it is difficult to multiplex and hence the amount of information per reaction is limited. In fact, in general qPCR protocols seldom use the full capacity of PCR machines since multiplexing of 4 or 5 targets requires significant optimization.

An alternative suite of PCR approaches opens the way for significantly higher multiplexing for both qPCR and SNP detection. In MNAzyme® qPCR, catalytic oligonucleotide complexes (MNAzymes) form in the presence of amplicons and cleave universal reporter probes. The bi-specificity of the MNAzymes, and the use of well-characterised universal probes sets (suitable for use with any group of targets), allows rapid development of highly specific and sensitive multiplex qPCR assays. Our group has recently demonstrated 20-plex qPCR whereby 20 genes were simultaneously co-amplification and individually detected in real time using 20 unique probes in 4 chambers.

PASS primers provide a cost-effective, flexible and robust method for multiplexed analysis of multiple genetic variants. PASS MNAzyme® qPCR allows the detection of SNPs and somatic mutations (point mutations, deletions and insertions) with sensitivity down to 1 in 1,000 (0.1%). The method is superior to ARMS PCR for multiplexing, particularly when mutations are present at the same, adjacent or nearby loci. As many as eight mutations at codons 12 and 13 of RAS have been successfully detected in a single well using three channels on a standard qPCR instrument. The technology is even more powerful when combined with newer instruments with higher multiplex capabilities such as Biocartis' Idylla™ platform. A multiplexed KRAS/BRAF assay, which detects 18 mutations, was evaluated using colon cancer and melanoma FFPE samples. Data from this 18-plex reaction showed >96% concordance with sequencing and its specificity was better than that reported for the singleplex Therascreen KRAS test.

In conclusion, the combination of PASS primer amplification and MNAzyme detection allows highly specific, robust analysis of multiple targets of clinical significance in convenient multiplex reactions which are compatible with standard qPCR machines.

Rapid High Sensitivity Monitoring of Tumor Genetic Changes for Targeted Therapy of Cancer

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Genetic variations in human cancer genes can determine whether a patient will respond to a specific therapeutic agent. For example patients with Non-Small Cell Lung Cancer (NSCLC) whose tumors carry mutations in the Epidermal Growth Factor Receptor (EGFR) can be responsive to tyrosine kinase inhibitors (TKI's) or their tumors can develop resistance to these therapeutics agents. Colorectal cancer patients that carry a single nucleotide change in Exon 2 of the KRAS proto-oncogene are unresponsive to ant-EGFR antibody therapy. Thus there is a need for a rapid, sensitive way to detect mutations in patient tumor derived DNA especially a minimally invasive technique that does not require surgery. QClamp is a real-time PCR method that can detect mutations in tumor derived DNA within a few hours from patient sample procurement. The technology allows the precise and sensitive detection of genetic variations without the need for any novel instrumentation.

Advanced Molecular Diagnostics (Poster 42 - 49)

Detection of DNA mutations with an electro-switchable PNA chip

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Background: The aim of personalized medicine is to select the ideal therapy for each patient, and the knowledge of an individual cancer patient's tumor characteristics and genetics has great importance in order to tailor the treatment. Colorectal cancer (CRC) is one of the most common cancers worldwide. The *KRAS* oncogene is mutated in approx. 35%-45% of CRCs, however it has been established that undetected KRAS mutant tumor subpopulations are frequently present in KRAS wild-type tumors. Testing for *KRAS* mutations to guide treatment with the anti-EGFR monoclonal antibodies cetuximab and panitumumab is now part of routine clinical practice. The purpose of this study was to develop a new method based on the switchSENSE technology to detect and specify KRAS mutations even in a high wild-type genetic background.

Methods: The switchSENSE principle: Electrically switchable DNA or PNA nanolevers are actuated at high-frequency on microelectrodes, while their orientation is monitored. The binding and thermal melting of analyte molecules modifies the switching dynamics in a characteristic way, providing information about the target. First, 116mer long DNA oligonucleotides (wild type and G12S mutant) were hybridized on a 14mer PNA chip. The melting transitions were characterized and the melting temperatures determined. Next, to determine the sensitivity of the assay for low quantities of mutant in wild type (wt) background, we mixed mutant and wt DNA oligonucleotides at wt/mutant ratios of 100/0, 90/10, 95/5, 99/1, 99.5/0.5 and 0/100. The conditions of the hybridization (temperature, NaCl concentration and additional wash step) were optimized so as to bind specifically only the fully complementary part of the mixed oligonucleotide probe on the PNA lever. Finally we repeated the described experiments with PCR-amplified dsDNA (wt and G12S mutant).

Results and Conclusions: According to our results a single mismatch has significant impact on the thermal stability of the PNA/DNA hybrids and induces a substantial decrease in the T(m) by about 10 degrees. Once the T(m)s were determined, the false-positive signal for a sequence with a single mismatch could be reduced to 0% at elevated hybridization temperatures of 45°C in combination with low NaCl concentration of 5mM and an additional low salt wash step at 45°C. The sensitivity for the fully complementary target sequence was maintained. Our mixing studies using G12S mutant and wild type oligonucleotide probes demonstrated that the electro-switchable PNA chip assay could reproducibly detect down to 0.5% mutant DNA in a wild type background. After optimization of the assay conditions and sample preparation we achieved to bind PCR products on the PNA lever. The determined melting temperatures were similar to those measured in the oligonucleotide experiments. To define the sensitivity of the assay for PCR-amplified dsDNA further experiments are planned.

Smartphone sized real-time PCR device

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Here we present a handheld device for real time polymerase chain reaction (rtPCR) for up to four samples. The core of the device is a virtual reaction chamber (VRC), consisting out of a 1 µL mineral oil droplet in which the aqueous sample droplet (100 nL) is suspended. The VRC is placed on top of a disposable, hydrophobic coated microscope cover slide, which in turn rests on a micro machined silicon heater. The heater consist out of four doughnut shaped structures, which each carry a resistive heater and temperature sensor made out of gold on the bottom side. Through the small thermal mass of the system (~2mJ/K) very fast heating rates (>20°C/s) can be achieved. Similar rates are obtained for cooling merely by passive cooling.

For detection, a miniaturized filter cube, similar to the ones found in fluorescence microscopes, was used. It consisted out of an excitation filter, a dichroic mirror as well as an emission filter. Each of the four samples had its own blue led for excitation as well as its own photodiode for detection. All functions are controlled by a printed circuit board (PCB) which includes a display for visualization of results. Furthermore a lock-in amplifier is integrated into the PCB filtering out signals with a predefined frequency and thus allowing fluorescent measurements in ambient light conditions.

For analysis commercial master mixes can be used. So far cDNA samples for the human transcripts HPRT and GAPDH as well as the avian flu (H7N9) have been used. A conventional thermal profile consisting out of a 20 seconds hot start at 95 °C followed by 40 cycles of 5 seconds denaturation (95°C), 5 seconds annealing (56°C) and 7 seconds extension (72°C) was used, resulting in a total analysis time of less than 12 minutes.

Open qPCR: The Design of an Open Source, $2000 Real-Time PCR Instrument

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Real-Time PCR is a powerful diagnostic technology, but the cost of existing instruments is typically beyond the reach of global health practitioners, undergraduate laboratories, and the emerging DIY biohacking movement. Here we present the design of Open qPCR, a low-cost Real-Time PCR instrument developed using rapid prototyping and open source technologies.

Open qPCR is a 16 well instrument with ramp speeds in excess of 5 C/s, and available with single or dual-channel detection. The device currently does presence/absence detection, relative quantification, and high resolution melt. The device uses a touch screen and web interface, supports ethernet/wifi/USB connectivity, and exports data in the standardized RDML format.

Open qPCR is released as open source hardware & software, and key design choices are discussed in this poster.

TaqMan® Rare Mutation Assays for QuantStudio® 3D Digital PCR System

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Detection and quantification of mutant alleles in tumor tissue allow for research disease monitoring and the research of drug efficacy. Detection of emerging secondary mutations in the same tumor tissue causing resistance to potential treatment will help guide decisions on future treatment plans. A less invasive research method than using tumor tissue is testing for the presence of mutations in circulating free DNA (cfDNA).

We created a research tool for mutation detection at a sensitivity level of 1% and below. This allows researchers to find correlation between types of mutations and types of tumors and determination of potential secondary mutations.

The tool combines TaqMan® SNP Genotyping Assays with digital PCR. A set of assays was optimized for use in digital PCR with the QuantStudio® 3D Digital PCR System. In digital PCR, partitioning the sample into many individual reaction wells facilitates detection and quantification of rare mutant alleles. TaqMan® SNP Genotyping Assays ensure reliable discrimination of mutant and wild-type allele.

Our initial set of 38 assays covers mutations commonly found in tumor tissues: BRAF V600E, mutations in EGFR exons 19, 20 and 21, KRAS codons 12 and 13, PIK3CA exons 9 and 20, and the JAK2 V617F mutations. All assays were wet-lab tested at a 10% mutation rate and a 1% mutation rate using mutant plasmid spiked into wild-type genomic DNA. Additionally, selected assays were tested at the 0.1% mutation rate using mutant cell lines spiked into wild-type genomic DNA.

Wet-lab results confirm that all assays showed superior performance discriminating mutant and wild-type alleles. Mutant alleles were successfully detected as low as 0.1%.

For Research Use Only. Not for use in diagnostic procedures.

Methods of Comparing Digital PCR Experiments

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The outcome of digital PCR (dPCR) experiments is usually the mean number of copies per partition (λ). The results are derived from measured data, which are an ordered (in one or two dimensions) list of positive partitions. The usual analysis assumes that the template molecules follow Poisson distribution among partitions. Based on that, already proposed approaches using the confidence intervals (Dube et a., 2008) or uncertainty of quantification (Bhat et al., 2009) allow a comparison of performed experiments. Bedsides these methods, there are also statistical tests which could be employed to compare the estimated mean number of copies per partition. However, their choice is limited by the specificity

of dPCR results. Measurements of digital PCR reactions often fall within a big data category (10k to 10Mio partitions/sample), where solutions useful for smaller data sets are no longer applicable. The typical multiple comparison framework requires post-hoc tests, where all experiments are tested if at least one of them yields a significantly different result. Then a pairwise comparison is performed. The other approach involves only the second step, i.e. the pairwise comparison between all experiments.

As an example, we compared a post-hoc testing procedure, the generalized linear model (GLM), and multiple proportion test (pairwise proportion tests utilizing Benjamini-Hochberg correction) over 150,000 simulated array dPCR experiments. Each simulation contained six reactions. Three of them had roughly the same amount of molecules per plate and other three were more abundant in 10 to 50 molecules. On average, 2.03 and 1.98 reactions were assessed to the wrong group by the GLM and multiple proportion test, respectively. To establish a stable and sensitive statistical procedure for dPCR experiments, we compared and evaluated available approaches based on GLMs, as well as proportion and ratio tests using different array and droplet dPCR setups. We considered not only theoretical parameters such as familywise error rate or false discovery rate, but also other important factors such as relative speed and computational hardness. The results of our findings were implemented in the open source ***dpcR*** package (Burdukiewicz and Rödiger, 2015) based on the statistical computing language ***R***.

The participation in the conference was funded by the KNOW Consortium.

Bhat, S. et al. (2009). Analytical and Bioanalytical Chemistry, 394(2), 457; Dube, S. et al. (2008). PloS One, 3(8), e2876; Burdukiewicz, M. and Rödiger, S. (2015), http://cran.r-project.org/web/packages/dpcR/index.html

High Resolution Melting Typing Approach For CTX-M β-Lactamases Differentiation

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CTX-M family of β-lactamase enzymes is the most common type of extended-spectrum β-lactamases (ESBLs). All known types of CTX-M enzymes can be assigned to the five phylogenetic groups, CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 or CTX-M-25. The High Resolution Melting(HRM)is very sensitive real-time PCR-based technology allowing precisediscrimination of homologous sequences. The proper fragment of bla_{CTX-M} gene sequence for the HRM analysis was selected. All tested samples were differentiated after an optimization of the target amplification and the HRM analysis. A new quick and robust HRM-based bla_{CTX-M} typing method was developed.

Engineered DNA Polymerases Tailor-Made For Your Needs

Ramon Kranaster

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myPOLS Biotec is actively pursuing research on DNA polymerases to shape their properties for advanced applications.

In the next months new DNA polymerases will be introduced by myPOLS Biotec that include:

- DNA polymerases resistant to known inhibitors (e.g., from blood or plants) allowing direct PCR without tedious sample preparations
- DNA polymerases enabling multiplex DNA and RNA diagnostics
- DNA polymerases that are tolerant to high salt conditions
- DNA polymerases allowing PCR from damaged DNA samples
- DNA polymerases that tolerate highly modified nucleotides
- Temperature sensitive DNA polymerases with intrinsic hot start function

As an example, we recently developed HiDi DNA polymerase that is an engineered DNA polymerase developed for approaches in which **hi**gh **di**scrimination between matched and mismatched primers is required (e.g., HLA analysis, allele-specific PCR (ASA) or methylation-specific PCR (MSP)). Whereas many DNA polymerases tolerate mismatched primers at least to some extent, HiDi DNA polymerase efficiently discriminates those and specifically produces amplicons in case of perfectly matched primer pairs only. This renders HiDi DNA polymerase the prime enzyme for SNP detections, HLA genotyping or the analysis of single CpG methylation sites in MSP. Based on HiDi DNA polymerase, we provide custom-made advanced and reliable qPCR mixes.

Furthermore, since HiDi DNA polymerase allows allele-specific PCRs directly from oral swabs without requiring DNA isolation or any other tedious sample-processing step, a Direct SwabPCR 2x Master Mix was established.

We also developed Volcano DNA polymerase – an engineered, extremely thermostable enzyme that has both, reverse transcriptase and PCR activity. Volcano DNA polymerase has a half-life at 95°C of >40 min and it facilitates “zero-step” RT-PCRs directly from RNA templates (without an isothermal reverse transcription step). These properties allow reverse transcription reactions at high temperatures, thus minimizing the problems encountered with strong secondary structures in RNA that only melt at elevated temperatures. Additionally, for those who wish to avoid expensive and time-consuming RNA extractions, we offer VolcanoCell that performs RT-PCR directly from primary cells or cell line suspensions.

Last but not least, all myPOLS DNA polymerases and their ready-to-use mixes are routinely tested to match highest quality demands in terms of activity, purity and functionality. All our DNA polymerases are produced exclusively in our laboratories in Konstanz, Germany. The established production is documented and scalable, yielding several milligrams of enzyme day by day.

Still missing the right DNA polymerase for your applications?

We are your expert for DNA polymerases and will assist you in establishing your approach using our wide knowledge on DNA polymerase engineering. With our expertise we tailor DNA polymerases for your needs - Contact us!

Biodistribution of Advanced Therapy Medicinal Products (ATMPs): Workflow of a typical study at a professional pre-clinical service provider

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ATMPs include gene and somatic cell therapy medicinal products. For such products, tumorigenesis, chromosomal instability and vertical transmissions in germline cells are potential risks. To address these issues, regulatory agencies worldwide require pre-clinical*ex vivo* biodistribution studies to evaluate the safety and toxicity of e.g. nucleic acid therapeutics. At IMGM, we design quantitative real-time PCR (qPCR) biodistribution studies investigating the persistence of the nucleic acid therapeutic in target tissues, the dissemination into non-target tissues and the expression in germ line cells. These studies are performed in the spirit of Good Laboratory Practice (GLP).

For our pre-clinical studies, we take advantage of the accurate absolute quantification of nucleic acids via qPCR using TaqMan chemistry on the state-of-the-art qPCR ViiA7 platform (Life Technologies).

A biodistribution study at IMGM starts with an extensive experimental design phase, where details concerning the goal of the study and requirements by licensing authorities are discussed.

The study is divided into two parts: In the validation study, the target-specific method is established, in the main study, the nucleic acid therapeutic is quantified in samples from various tissues. Both studies are first described in a detailed study plan and concluded with a study report as required by GLP regulations.

In the validation study, calibration curves for absolute nucleic acid quantification are established to validate a quantification range. Additionally, acceptance criteria for quality control (QC) parameters like accuracy and precision, sensitivity and selectivity, recovery rate and stability of the nucleic acid therapeutic are determined to ensure that the molecule is detected and quantified specifically. The main study starts when the validation study report is approved by quality management and the customer.

In the main study, the nucleic acid therapeutic is quantified in all study samples in triplicates using the defined calibration curve and acceptance criteria for QC parameters from the validation study. Every qPCR run includes calibration curve and additional QC samples in triplicates. All data are documented thoroughly, presented in a final report, and archived in the spirit of GLP.

A biodistribution study at IMGM provides all information required by licensing authorities about the nucleic acid therapeutic and its quantitative biodistribution in various tissues.

Molecular Diagnostics in Agriculture, Veterinary Medicine, Food & Environmental Science (Poster 50 – 69)

Concentration of Pathogenic Viruses from Seawater Samples using Monolithic Chromatographic Supports and Downstream Detection using Quantitative PCR

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Monitoring of the fecal contamination resulting from anthropogenic activity in coastal water and seawater is essential to ensure the safe use of these water bodies for recreational use, aquaculture, and to assess any biogeochemical changes in the coastal marine environment. Although assays for detection of conventional fecal coliform indicators such as *Escherichia* coli and intestinal enterococci are established, evaluation of the presence of the more persistent viral pathogens in seawater needs to be standardized. Since exposure to as few as 10-100 particles of enteric viruses is known to cause disease, there is a need to develop a rapid and sensitive diagnostic method. The quantitative PCR (qPCR)-based assays developed can detect as few as 10^4 enteric viral particles per ml of environmental water samples. However, the detection of these viruses at the levels expected in the coastal marine environment, and the potential presence of PCR inhibitors in these water matrices precludes the use of an initial concentration step. CIM (convective interaction media) methacrylate monolithic columns in conjunction with qPCR assays have been successfully deployed for detection of enteric viral pathogens like rotavirus, norovirus, Hepatitis A and calicivirus from different fresh water, surface water and wastewater matrices. CIM was also successfully used for virus target enrichment before NGS experiments in diverse samples, such as stool and plant tissue. Same strategy may be applied to marine waters, upon proper optimization.

In this study we evaluate the application of CIM monolithic chromatography to concentrate pathogenic enteric viruses from seawater using rotavirus as a model. To optimize the binding of rotavirus, different strong cation-exchange (SO3), and strong (QA) and weak (DEAE) anion-exchange chemistries, as well as different pH and salinity gradient conditions were tested using disk format (bed volume 0.34ml). The viral concentration in fractions was quantified by one-step reverse transcription qPCR (RT-qPCR). We present our results on the most effective conditions to concentrate rotavirus at high salinity using CIM monolithic support. Furthermore, the extension of our protocols to other enteric viruses and the scaling-up of the CIM monoliths to handle greater water volumes represents an attractive preparative technique for downstream qPCR and NGS based detection of viral pathogens in seawater.

Evaluation And Quantification Of The Lactogenesis Of Primary Bovine Epithelial Cells *In Vitro* - 3D Cell Culture Model Of The Bovine Mammary Gland.

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The bovine mammary gland and its functionality is of great interest not only to the dairy industry but also to the food and pharmaceutical industry. Those industries often equate the bovine mammary gland with a huge bioreactor that is useful in terms of the production of transgenic therapeutic proteins and of course for the optimization of the milk composition in terms of human nutrition. Therefore it is important to unravel the details of milk protein production and the involvement of the gland and their functional subunits (e.g. cells) in the immune response of the bovine mammary gland. To create a new more *in vivo*-like model of the bovine mammary gland, we established a three dimensional cell culture model of primary bovine epithelial cells (pbMEC) extracted from milk in our institute. The isolation of pbMEC from bovine milk, which was established in 1990 (Buehring, 1990), is a routinely used cell culture technique in our institute. The pbMEC cultured on Matrigel™, a soluble extracellular matrix like formulation, enabled us to induce the milk protein production *in vitro*. We used gene expression measurements and HPLC analytic to evaluate the milk protein production *in vitro*. The mimicry of the extracellular matrix with Matrigel™ promotes the differentiation process of the cells and allows the recapitulation of *in vivo*-like characteristics, like the formation of tubular- and alveolar structures which are similar to those in the mammary gland parenchyma. The *in vitro* stimulation of pbMEC with the lactogenic hormones prolactin and hydrocortisone induced lactogenesis in several primary bovine epithelial cell lines after the first subculture of the freshly isolated cells. We could proof the induction of lactogenesis through analysis of the gene expression levels of the two whey proteins α-lactalbumin and β-lactoglobulin and the milk proteins α-S1-casein, α-S2-casein, β-casein and κ-casein in the cell lysate. In addition we quantified the milk proteins secreted in the cell culture supernatant during the induction process, using reversed phase HPLC analytics. We could show that pbMEC are able to express and secrete milk proteins *in vitro* after induction with lactogenic hormones over a time period of 4 days. Summarizing this we assume that the cultivation of the pbMEC in 3D itself and the combination of 3D - cell culture and supplementation with lactogenic hormones induce and maintain the transcription of milk proteins in polarized pbMEC. All in all the 3D cell culture model of pbMEC is a promising approach for future research of physiological regulatory mechanisms in the mammary gland. There is a large scope of applications for this 3D cell culture model as it can also be used to investigate the interaction of immune cells and pbMEC during the defense of pathogens.

The Fungal Nutritional ENCODE Project: Exploring Fungal Carbon Perception in the Model Filamentous Fungus*Neurospora crassa* by a Large Scale RNA-Seq Approach

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Fungi affect our lives in multiple ways. They are important organisms for enzyme production and the deployment of second generation biofuels, but on the other hand can destroy important feedstocks, foodstuff and energy resources. With a better understanding of how these fungi degrade biomass, it should be possible to improve the economics of production processes, increase enzyme yields or prevent the decomposition of valuable materials such as construction wood.

The filamentous ascomycete *Neurospora crassa* (*N. crassa*) is a model organism well suited for deciphering the complex biomass degradation processes. It is an organism with simple growth requirements that is proficient at degrading lignocellulose as sole carbon source. Moreover, a variety of molecular, genetic and biochemical techniques have been developed for *N. crassa*, including a publically available full genome deletion strain set. Therefore, *N. crassa* is a perfect candidate for the utilization of systems analysis to elucidate the molecular networks governing the perception of biomass by fungi.

To generate a comprehensive gene regulation model of *Neurospora* during biomass degradation, we have initiated the Joint Genome Institute (JGI) Community Sequencing effort “The Fungal Nutritional ENCODE Project”. By using RNA-Seq, the transcriptional response of *N. crassa* to a variety of carbon sources (mono-, oligo-, and polysaccharides up to complex biomass) as well as to variations in sulphur, phosphate and nitrogen availability will be recorded. With the acquired data it should be possible to create a high-resolution model of the gene regulation of *Neurospora* at a genome-wide scale. This will, in the end, enrich the genome annotation of*N. crassa*, and in extension, the genomes of other filamentous fungi. More importantly though, through the accumulation of the transcriptomic data it will be possible to unravel how fungi recognize a possible substrate and, as a consequence, change their metabolism to effectively utilize it.

What’s The Difference Between A White And A Red Strawberry? A Comparative Approach To Gain Insight Into Expression Of Anthocyanin Biosynthesis Pathway Genes

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During the last decade, the worldwide production of strawberries increased by 29 % up to 4.5 million tons. That makes the strawberry one of the most popular fruit crops worldwide. Along with the sweet flavor and the numerous health-promoting ingredients, much of the attractiveness is founded in the deep red color caused by anthocyanin pigments. In contrast to the red fruited woodland strawberry, there are also varieties that have white fruits even when they are fully ripened. Interestingly, the flavor of these white fruits is as diverse as that of red fruits, but there must be a distinctive difference in gene expression level that causes such a dramatic change of color. In order to gain a deeper insight into the regulation of anthocyanin biosynthesis we performed a comparative transcriptome analysis of one red-fruited and two white-fruited woodland strawberry varieties by next generation sequencing. Free software solutions were used, to map the Seq-data to a strawberry reference genome and to perform differential gene expression analysis. In parallel, the anthocyanin level of all tissues was determined via LC-UV-ESI-MSn. The expression of single genes will be validated by quantitative PCR.

High-throughput Gene Expression Analysis In Pigs As Model For Respiratory Infections

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Influenza A virus infections have great impact on human health and welfare and significant resources are linked to influenza epidemics due to excess hospitalizations and lost productivity. Up to 15% of the human population is affected when Influenza *spreads around* the*world in* seasonal *epidemics (WHO)***.**

Animal models are essential in understanding the mechanisms involved in human infectious disease and for the development of effective prevention and treatment strategies. It is increasingly realized that large animal models like the pig are exceptionally human like and serve as an excellent model for disease and inflammation. Pigs are fully susceptible to human influenza, and have been demonstrated to be involved in influenza evolution and ecology. Pigs share many similarities with humans regarding lung physiology and innate immune cell infiltration of the respiratory system and thus seem to be an obvious large animal model for respiratory infections.

By using Fluidigm high throughput RT-qPCR, this study aimed at providing a better understanding of the involvement of circulating non-coding RNA and innate immune factors in porcine blood leukocytes during influenza virus infection. By employing the pig as a model we were able to perform highly controlled experimental infections and to study changes of symptoms, viral titer, and expression of microRNAs/mRNAs as the influenza infection progresses in time, generating information that would be difficult to obtain from human patients.

Pooling of fecal and environmental samples for the detection of *Salmonella* spp. by qPCR

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Several studies have evaluated the use of qPCR for the detection of *Salmonella* spp. in fecal and environmental samples. The purpose of this study was to evaluate the pooling of feces and environmental samples following a selective enrichment culture step for the detection of *Salmonella* spp. by qPCR. Equine fecal and environmental samples were collected at veterinary hospitals, inoculated into selenite broth and incubated overnight, then processed for DNA purification. *Salmonella* spp. qPCR assay targeting the *invasion A* gene was performed. The pooling strategy of collected samples incubated in selective enrichment broth was able to detect all culture and individual PCR positive samples. This strategy appears to be cost- and time-effective in a hospital environment with a low prevalence for *Salmonella*spp.

Strategies To Monitor Extraintestinal Pathogenic *E.coli* ExPEC

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ABSTRACT:

In the USA and in Germany occasional outbreaks of highly pathogenic E.coli strains (EHEC/HUS-2011) are correlated to the unprofessional use of manure. We detected phylogenetic groups (B1, B2, D and A) by standard qPCR using taqman-probes[1]. Bovine animals do shed a numerous amount of E.coli belonging to group B1. We quantified the subclasses to check manure (to improve manure production), soils and plant foliage. The distribution (presence/absence) of a range of virulence factors varies among strains of the different phylogenetic groups[2]. Extraintestinal pathogenic E. coli (ExPEC) are responsible for a spectrum of invasive human and animal infections, often leading to

septicemia[3]. Unlike non-pathogenic commensal and intestinal pathogenic E. coli, ExPEC derive predominantly from E. coli phylogenetic group B2, and to a lesser extent from group D[4]. To guarantee maximum security during the cultivation of vegetables for human consumption we as well quantified about 20 published virulence factors of group B1, B2, and D positive samples, to exclude occasional delivery of microbial contaminated vegetables. In addition genes related to antibiotic resistance ESBL (Extended Spectrum b-lactam resistance) as well as the NDM-1,2 (New Delhi metallo-b-lactamase) were detected. The threshold to restrain vegetables from the marked is set at 0 KBE/cm^2 for foliage.

The marketing of vegetables contaminated by pathogenic E.coli can be avoides by simple qPCR methods discriminating phylogenetic groups (B1, B2, D and A). To decide to restrain vegetables from the marked about 25 common virulence factors can be quantified.

Small RNA sequencing in milk of cycling and pregnant cows

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The most critical phase of pregnancy is the first days and weeks after insemination. During this period about 50% of lactating cows suffer embryonic loss prior to implantation. This is a high economical deficit for farmers. An early detection of pregnancy could allow the discrimination between fertilization failure and early embryonic losses. Milk is an optimal sample material as it is obtained daily and non invasively from the animal. We therefore want to establish a new pregnancy test system based on the discovery of small RNA biomarkers derived from somatic milk cells, mainly leucocytes (LC), and skim milk (SM) of cows.
In this study milk samples were taken on days 4, 12 and 18 of cyclic cows and after artificial insemination, respectively, of the same animals. The milk was centrifuged, fat layer removed and SM samples frozen at -20°C. The remaining cell pellet, including LC, was washed, collected and stabilized in Qiazol at -80°C. Total RNA was extracted and RNA integrity assessed. Library preparation of LC and SM samples from n=6 animals was done with NEBNext® Multiplex Small RNA Library Prep Set and the samples were sequenced in the HiSeq 2500 from Illumina. The resulting dataset was then processed bioinformatically to obtain the measured miRNAs and piRNAs aligning to miRBase for mature bovine miRNAs and to the human genome database with piRNA properties, respectively. The resulting readcounts were normalized using DeSeq and analyzed with DeSeq R-script and GenEX software using a principal component analysis (PCA) and a dynamic PCA.
The RNA integrity was lower in SM with a RIN average of 2.3 compared to 6.1 in LC.
15 mio. reads/sample were measured and of these 3% mapped to miRNAs or piRNAs respectively. A high number of reads was shorter than 16 nt. Interestingly, miRNAs were more present in LC than in SM while piRNAs are more present in SM (2 or 1% in LC, 1 or 2% in SM, respectively). 133 miRNAs and 75 piRNAs with more than 50 reads were found in LC and 95 miRNAs or 78 piRNAs respectively were found in SM.
LC and SM samples as well as animals differentiated in a PCA, in contrast to pregnant or cyclic states. However changes between pregnant and cyclic stages were found in LC for a number of small RNAs with a tendency of up-regulation (let-7i, miR-186, miR-25, miR-221, miR-106b, miR-146b, miR-93 and piRNA-41209) or down-regulation (miR-200c, miR-200b, miR-193a) in pregnant animals from day 4 to 18. The fold regulations of these changes were comparable between animals but occurred on different levels of expression.
No changes of small RNA could be found in skim milk samples. This may be either explained by the low RNA integrity in these samples or because the uterine information is transferred preferably over cellular elements and less over circulating nucleic acids. The small RNA biomarker candidates will further be analyzed using RT-qPCR and validated on independent samples.

In-house Validation of DNA Extraction Method from Flour Samples and Rye, Maize and Oat Flour contents in Wheat Flour Based Composite Breads

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The extraction of high quality DNA is a crucial technique for food samples in molecular biology. The quality of DNA determines the reliability of downstream process such as real time PCR. However, the published methods for the extraction of food samples DNA were usually inefficient for high-throughput, large scale molecular techniques to analyse gene expression or quantification patterns. In this paper, we reported a high quality DNA extraction protocol from flour samples and rye, maize and oat flour contents in wheat flour based composite breads. Food matrices such as flours and the wheat-based composite breads supplemented with rye, maize or oat revealed to be difficult matrices, since large amounts of compounds tend to co-purify with DNA, even adopting well established extraction protocols. Yield and purity of DNA extracts were used to demonstrate DNA quality and quantity. On the other hand the absence of co-extracted compounds in a DNA sample impairing the efficiency of the PCR reactions and leading to a delay in the onset of the exponential phase of the amplification profile. The extracted DNA was further used for taxon specific PCR analysis through Real Time PCR. The results indicate that DNA was of good quality and fit for real time PCR. Firstly, genomic DNA was extracted from all flour and composite bread samples according to the CTAB-based method reported in ISO 21571: 2005. As an alternative method, foodproof sample preparation kit (Biotecon Diagnostics) was used for DNA extraction form all samples. We explained that high throughput DNA extraction protocols can be used to extract high quality DNA from flour and wheat flour based composite bread samples for qualitative and quantitative real time PCR analysis. For each method, the greatest average DNA quantity and purity index was from flour samples, while bread samples showed greatest DNA quantity and purity index using the CTAB DNA extraction method. The kit method provided a great PCR success rate.

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Development of a Molecular Method for the Detection of Hepatitis E Virus (HEV) in Pancreatin.

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Pancreatin is a mixture of several digestive enzymes produced by the exocrine cells of the swine pancreas. It is composed of amylase, lipase and protease. Pancreatin is employed as drug in humans to treat pancreatitis and other conditions involving insufficient pancreatic secretions. The drug is produced from porcine pancreases and this poses the risk of zoonotic transmission of pathogens, primarily hepatitis E virus (HEV). HEV is a small, non-enveloped RNA virus that causes acute hepatitis in humans. Notably, about 1% of the starting material (homogenized tissue) used for pancreatin production is constituted by small intestine, which is a major extrahepatic site of HEV replication in pigs.

The aim of the study was to develop a method for detection and quantification of HEV in pancreatin. HEV can not be easily grown in cell culture by conventional cell culture and therefore an approach based on quantitative RT-PCR (RT-qPCR) was selected. This entailed the use of a non-HEV internal (IC) control to monitor RNA extraction efficacy and the production by *in vitro* transcription of HEV synthetic RNA as a reference to account for the efficacy of reverse-transcription. A lab-adapted pancreatin production process was set up to mimic the event of contaminated batches of porcine pancreas/small intestine. Extraction efficiency from different matrices (faeces, activated frozen pancreas homogenate and pancreatin) was assessed by HEV and IC standard curves. The method has been evaluated by experiments in which HEV (from naturally

infected pig faeces) was spiked both in the starting material (porcine pancreas/duodenum homogenate) and in pancreatin. Our approach allowed evaluating the capability of the method to detect HEV in pancreatin and to simulate HEV contamination of porcine tissues for detection by RT-qPCR after processing.

In conclusion, a method for the detection of HEV in pancreatin has been developed with the possibility to be employed at the top of the production process (on porcine pancreas homogenate = starting material) with quantitative performance or on the final product (pancreatin) as a qualitative assay. Both strategies will ensure a reliable detection of HEV genome in pancreatin. On the basis of the current knowledge, the risk of HEV presence in the homogenate for pancreatin production, even though appears to be low, can not be completely ruled out and testing for HEV following the precautionary principle should be the guiding rule.

Effects of prostaglandin F2alpha on some local luteotropic and angiogenic factors in corpus luteum during induced luteolysis in cow

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In ruminants, luteal regression is stimulated by episodic release of endometrial prostaglandin F2alpha (PTGF). However, the acute effects of PTGF on known local luteotropic factors (oxytocin-OT and its receptor-OTR and progesterone and its receptor-PR), the principal angiogenic factor VEGFA and the vasoactive factors (angiopoietin 1-ANPT1 and angiopoietin 2-ANPT2) have not been studied in detail. The aim of this study was therefore to evaluate the tissue concentration of these factors during PTGF induced luteolysis. In addition the mRNA expression of PR, OTR, ANPT1 and ANPT2 was determined at different times after PTGF treatment. Cows (n=5 per group) in the mid-luteal phase (days 8-12, control group) were injected with the PTGF analogue (cloprostenol) and CL were collected by transvaginal ovariectomy at 0.5, 2, 4, 12, 24, 48 and 64 h after injection. The mRNA expression was analyzed by a quantitative real-time PCR, and the protein concentration was evaluated by enzyme immunoassay or radio immunoassay. Tissue OT peptide and OTR mRNA decreased significantly latest after 2 h followed by a continuous decrease of OT mRNA. VEGFA protein decreased already after 0.5 h. By contrast ANPT2 protein and mRNA significantly increased during the first 2 h followed by a steep decrease after 4 h. The acute decrease of local luteotropic activity and acute changes of ANTP2 and VEGFA suggest the important role of these local produced factors in modulation of vascular stability in CL that may be a key component during functional luteolysis in cow.

Digital PCR For Quantification Of *Listeria monocytogenes*

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The digital PCR (dPCR) is the third *generation* of PCR and it represents a new approach to nucleic acid detection and quantification based on molecular counting. It employs components of traditional quantitative PCR (qPCR), but the biggest difference is that reactions are partitioned into thousands of compartments for amplification. The goal of dPCR is for each compartment to contain none or one target molecule. After the amplification and data collection, the ratio of positive to negative compartments can be used to calculate the absolute number of target molecules in the original sample without reference to standards or endogenous controls.

dPCR extends qPCR capabilities for advanced applications like high-precision copy number variation, absolute quantification of bacterial and viral loads, reference and standard quantification and also GMO detection.

The objective of our preliminary study was to test an already validated qPCR assay for *Listeria monocytogenes* with the new platform QuantStudio 3D (Life Technologies). The same assay was used with both QuantStudio 3D and ABI 7500 Fast instruments. We compared both platforms in terms of efficiency, linear range and sensitivity of quantification. Finally, DNA from poultry meat samples was used and the samples were analyzed using the new dPCR approach. We demonstrated the usefulness of dPCR for the exact quantification of standards, which can be subsequently used for quantification of contaminated poultry meat samples with qPCR.

Postprandial recovery of colostral exosomes and RNA in calf plasma

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Colostrum is a nutrient-dense biofluid high in immunological factors, bioactive molecules and extracellular vesicles (EVs) such as exosomes. It plays a crucial role in early calf development, providing nourishment, immunological protection and a variety of signaling molecules. EVs are important mediators of intercellular communication and we therefore looked into the potential transfer of EVs, more specifically exosomes, from colostrum to neonatal calves.

Blood was drawn from calves pre colostrum feeding and at various postprandial points. Low-speed centrifugation was then employed to separate plasma and skim colostrum collected at the initial feeding. Exosomes were isolated from colostrum and plasma by differential ultracentrifugation and density gradient centrifugation and further analyzed by western blotting for CD63 and MFGE8 as a general and milk-specific marker, respectively. To assess EV populations, Nanoparticle Tracking Analysis was used for the evaluation of particle count and diameter in distinct gradient fractions. Additionally, protein quantification (BCA assay) and RNA analysis (RT-qPCR) were also performed in exosome-containing fractions.

Colostral exosomes feature a slightly higher density than exosomes from adult bovine plasma; floating in 40 – 50 % sucrose (1.18 – 1.23 g/ml), as opposed to ≤ 30 % sucrose (≤ 1.13 g/ml). In calf plasma, interestingly, the vast majority of vesicles is also found in the higher density fractions. After the initial colostrum feeding post-partum, the calf's particle count in 40 – 50 % sucrose, but not 30 % sucrose, increases significantly. This indicates a transfer of colostrum-derived exosomes to the calf's circulation. Consistently, total exosomal protein in calf plasma increases linearly over the first days post-feeding. Immunoblotting further supports these findings: while plasma exosomes from adult animals and neonatal calves are MFGE8-deficient, the milk-specific MFGE8 signal is found in calf plasma exosomes as early as 6 h post-feeding. Furthermore, a significant increase in milk-specific micro RNAs (e.g. miR-200b, miR-148a, miR-30a-5p) in calf plasma post colostrum feeding was shown by RT-qPCR; also suggesting an uptake from milk.

In summary we found that exosomes from bovine plasma and colostrum feature distinct flotation densities. Also, flotation densities between exosomes isolated from plasma of adult animals and neonatal calves seem to differ. Both protein and RNA markers as well as particle density in the calf's circulation indicate that exosomes from colostrum are taken up in early calf development.

Estimation of Measurement Uncertainty in Quantitative Analysis of Roundup Ready® Soybean and Lectin Gene using Plasmid Reference Material

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Quantification of genetically modified (GM) food, feed and seed based on DNA with Real Time PCR (RT-PCR) has become routine analysis in food control laboratories after the recently introduced labeling threshold for genetically modified organisms (GMOs). For these analyses, precise thresholds are set by legal regulations which makes measurement uncertainty an important aspect in the molecular biology methods. In order to quantify the amount of GM in the food, feed and seed calibration has to be generated with Certificated Reference Materials (CRMs) by RT-PCR method. In this study, two different specific designed plasmid reference materials for Roundup Ready® soybean commonly used as GM and Lectin as a endogenous gene of soybean are chosen as calibrant DNA instead of CRMs. RT-PCR calibration in terms of copy number of plasmid reference material and quantification of GMOs were performed with SYBR green I technique. Measurement uncertainty of calibration data was estimated and calibration curves were drawn which resulted in the quantification of GMOs. Results demonstrated that plasmid reference materials are effective alternatives to CRMs.

Incidence And Detection Of Beak And Feather Disease In Psittacine Birds In The UAE

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Beak and feather disease is caused by Circovirus, which affects actively growing beak and feather cells of avian species. A Taqman probe based Real-Time PCR assay was used to detect the presence of the viral genome in psittacine birds in UAE. Here, we report the incidence of beak and feather disease in different species of psittacine birds. The highest rate of positives were found in African grey parrots (57.7%), followed by parakeets (43.8%) and macaws (18.1%). The sensitivity of the assay was found to be very high with detection limit of upto 8.9 *10^{-6} ng of DNA in the sample. The Taqman assay is a quick, reliable and sensitive detection method that has been instrumental in identifying this disease that was not previously reported in the region.

Influence Of Milk Procession On miRNA Stability And Its Relevance In The Immune System

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Raw milk consumption especially during childhood was shown to positively affect a number of respiratory diseases (e.g Asthma and atopic sensitization). Since raw milk also bears the potential risk of life-threatening infections, identification of these beneficial components and their preservation in industrial processed milk are in high demand. The aim of this study was to assess the degradational effect of different procession types of bovine milk and to screen them for specific miRNAs in order to investigate possible interferences of miRNAs with genes and pathways implied in the pathogenesis of respiratory infections and allergies.

Milk samples were collected from different farms and subjected to the common procedures found in industrial processing: Separation of milk fat, homogenization, pasteurization, heat treatment for extended shelf life and ultra-high temperature (UHT) treatment. In addition milk samples were boiled to simulate normal household conditions, resulting in 8 distinct milk fractions per farm. Total RNA was extracted and RNA concentrations as well as purity were assessed before subjecting all samples to small RNA NGS. After read data filtering of unwanted sequences, mapping was performed on the most recent miRBase data for mature human miRNA. Differential expression was verified by RT-qPCR for several miRNAs and the potential impact of miRNAs on human health was evaluated by comparing them to experimentally verified miRNA targets of the DIANA-TarBase v6.0 with a focus on 139 rhinitis, asthma and allergy candidate genes.

RNA content between milk fractions varied greatly with heat treated samples showing the lowest concentrations (ng/µl: ESL 8.89, UHT 12.00, boiled 12.30) and raw milk samples showing the most (46.33 ng/µl). NGS data analysis revealed the presence of 1796 miRNAs with 155 showing a sufficient abundance with more than 50 reads per 10 million. While different farm exposures proved to have no influence, milk processing affected the composition of miRNAs significantly. High temperature treatment resulted in a significant reduction of miRNA reads (5.51 fold) which was further supported by the absence of a distinct 22nt peak in the read length distribution across all milk types. PCA analysis separated milk samples well with the first PC distinguishing between high heat treatments and moderately-treated samples and the second PC showing the difference between fat and non-fat samples. Clustering in PCA was dominated by human miRNA 148a-3p as the major milk miRNA and to a lesser level by let-7a-5p, let-7b-5p, let-7f-5p, 22-3p, 26a-5p, 30a-5p and 200c-3p.

Evaluation of targets of significantly regulated miRNAs in UHT lead to a high number of mRNAs involved in the pathology of allergies as well as mRNAs involved in the immune response to respiratory infections. This indicates that beneficial effects of raw milk consumption can be in part explained by their still intact miRNAs especially compared to industrial processed milk.

New analytical approaches in GMO analysis for the detection of 'stacked events'

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In recent years, genetically modified plants (GMP) have increasingly been combined through conventional crossing resulting in so called 'stacked events'. These 'stacked events' have gained importance worldwide as pointed out by a rapid increase in cultivation area from 3.2 million hectares (2000) to 51 million hectares (2014).

In the European Union (EU), 'stacked events' must be authorized in order to be imported or cultivated, regardless to the authorization status of their parental lines. As part of the authorization process, the producer is obliged to provide reference material and a specific detection method to identify this GMP. Currently, 30 'stacked events' are authorized in the EU, however, specific detection methods and certified reference material are usually available for the parental lines only. There are two exceptions: a stacked maize event and a stacked cotton event, for which also reference material is available. Specific detection methods to distinguish a 'stacked event' from its parental lines are not on hand.

The current general procedure for identifying GMP is to detect the junction sequence of the plant genome and the inserted transgenic DNA fragment (event-specific detection). However, by way of an event-specific detection, 'stacked events' cannot be distinguished from its parental lines as the border sequences of the inserted DNA and the surrounding host genome remain unchanged after conventional crossing.

Hence, the European Network of GMO laboratories (ENGL) established a working group in order to identify new analytical approaches to discriminate between 'stacked events' and their parental lines. Two promising approaches are the detection of molecular markers in the genetic background of GMP or the detection of SNPs/InDels in transgenic DNA inserts of the 'stacked events'. This can be accomplished by applying the next generation sequencing (NGS) technology.

In our project we follow the second approach by analyzing transgenic DNA inserts of different 'stacked events' and their parental lines by NGS (in cooperation with the Gene Center of the University of Munich). We used an automated DNA extraction method to isolate DNA from different 'stacked events' (MON863×MON810, MS8×RF3, MON89034×MON88017, NK603×MON810, MON89034×MON88017) and their parental lines. Primers were designed to amplify the respective insert using a high-fidelity Q5 DNA Polymerase. Amplicons were purified and underwent NGS analysis. Preliminary results are presented.

Real-time PCR Based Concept for Monitoring of Genetically Modified Oilseed Rape (*Brassica napus L.*) along Transport Routes

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Regarding oilseed rape, no GM event is currently authorized for cultivation in the EU, while the import and the processing of certain GM rapeseed lines are allowed. However, the occurrence of genetically modified (GM) rapeseed in the environment might result from seed loss during transport or processing of imported oilseed rape seeds both as pure product or contaminant of conventional products. In order to obtain information on the presence of feral GM oilseed rape populations in Bavaria, a monitoring concept was elaborated. Since Bavaria is free of GMO cultivation, feral populations of GM oilseed rape are most likely to occur along transport routes for rapeseed. In terms of an efficient monitoring, transshipment stations, inland ports and rapeseed processing plants were chosen as primary sampling sites. Initial samplings were used to optimize the analysis workflow including sample pretreatment and DNA-extraction, adaptation of a multiplex screening PCR for rapeseed leaves from feral populations of approved GM oilseed rape lines and the succeeding event specific real-time PCRs. In addition, the use of lateral flow membrane stripes was evaluated. We present data concerning the development and application of a real-time PCR based analysis workflow for monitoring GM oilseed rape along transport routes.

Molecular identification and Physiological characterization by Flow Cytometry of bacteria and yeastsproducing polygalacturonase (PG) isolated from fermented cassava microbiota

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The Flow Cytometry is a method increasingly used in food industries for rapid characterization of microorganisms for the production of starters or probiotics. In this study, the FC was used after the molecular identification of the microorganisms and microplate culture in three glucose concentrations (1; 5 and 10g /l) and at temperatures ranging from 30 to 45°C, to characterize these strains and analyzing the physiological and morphological changes of the cells after heat stress. Thus, 2 yeasts strains (*KSY4* and *KSY5*) and 2 bacteria strains (*KSB30* and *KSB32*) producers of polygalacturonase and isolated from fermented cassava, were used. The results that we obtained, showed that the yeasts strains belonged to the species *Saccharomyces cerevisiae*; and bacteria also are close to the species *Bacillus amyloliquefaciens*. The FC analysis after staining of the cells with PI and CFDA revealed the presence of a strong sub-population of VNC cells (approximately 80%) than the cells having retained the membrane integrity in both species at 42°C. A new cell shape was detected in FC during cultivation of *S. cerevisiaeKSY5* at 30°C. Among yeasts, one of strains studied is able to withstand 45°C and with a low end of the culture in mortality. On the other hand, *Bacillus amyloliquefaciens* well known for its ability to produce endospores in hostile growing conditions would be able to grow well at the same temperature, with a low mortality rate in a medium containing a low proportion of glucose (1g/l). These results show the importance of this approach and provide an opportunity to characterize a single cell of microorganisms for perform and optimized the bioprocesses.

***Keywords*:** cassava; *Bacillus amyloliquefaciens; Saccharomyces cerevisiae*; Flow Cytometry; polygalacturonase

Sequence Differences In Corynebacterium Pseudotuberculosis Isolated From Caprine And Camel Species

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Corynebacterium pseudotuberculosis is an intracellular pathogen of veterinary and economic importance and mainly affects sheep and goats causing abscess in the lymph node, leading to caseous lymphadenitis (CLA). Camels are also known to be affected by the disease to a much lesser degree with the bacterium causing a different kind of lesion. Isolates of *C. pseudotuberculosis* from 63 camels, 4 caprine and 2 oryx species were amplified using primers targeting a 954 bp region in the virulence gene phospholipase D. Sequencing of the amplicons revealed that all the isolates from camel had six distinct nucleotide variations from the caprine and oryx sequences. Further investigation is required to see if these mutations correlate to the variation in susceptibility and type of the disease observed in the camel population.

qPCR Data Analysis (Poster 70 – 73)

The *dpcR* Package – a Framework for Analysis and Visualization of Digital PCR Experiments

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Digital PCR (dPCR) is a precise technology which may enable absolute quantification of nucleic acids. Hardware platforms partition reactions using water-in-oil emulsions or nano-chamber-based approaches [1]. Commercial software tied to a specific system has been available for users. We have built what we believe to be the first unified, cross-platform, dMIQE compliant, open source (GPL-2) software frame-work for analyzing digital PCR experiments. Our frame-work, designated ***dpcR*** [2], is targeted at a broad user base including end users in clinics, academics, developers, and educators.

Our software can be used for (I) data analysis and visualization in research, (II) as software frame-work for novel technical developments, (III) as platform for teaching this new technology and (IV) as reference for statistical methods with a standardized nomenclature for dPCR experiments.

dpcR is free of charge and available from the Comprehensive R Archive Network (CRAN). It builds upon the ***R*** software environment for statistical computing and graphics. Our ***dpcR*** frame-work includes tools for data analysis, data visualization, simulation and data exchange. Features such as functions to estimate the underlying Poisson process, methods from peer-reviewed literature for calculating confidence intervals based on single samples as well as on replicates, a novel Generalized Linear Model-based procedure to compare digital PCR experiments and a spatial randomness test for assessing plate effects have been integrated. Customized implementations of the ***dpcR*** package cover remote browser applications and a standalone desktop application, both including a graphical user interface.

In conclusion, ***dpcR*** provides means to understand how digital PCR works, to design, simulate and analyze experiments, and to verify their results (e.g., confidence interval estimation), which should ultimately improve reproducibility. Our frame-work is suitable for teaching and includes references for an elaborated set of methods for dPCR statistics. Due to the plug-in structure of the software it is possible to build custom-made analyzers.

[1] Morley AA., *Biomolecular Detection and Quantification* (2014), **1/1**, 1-2

[2] Pabinger and Rödiger et al., *Biomolecular Detection and Quantification* (2014), **1/1**, 23-33

The reliability of two different calibration of 2^{-DDCt} method for analysing relative gene expression in real time pcr

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Relative quantification associates the PCR signal of the target gene in a treatment group to an untreated control. The 2^{-DDCt} method is a most commonly used to analyse the relative changes in gene expression from real-time quantitative PCR experiments. The aim of this study is to investigate the reliability of two different calibrations of the 2^{-DDCt} method.

Methods: Primary human PDLSC and gingival fibroblasts (GF) were cultured for a period of up to 28 days in normal and osteogenic differentiation media. The expression of periodontal associated marker such as PLAP-1 was analysed using qRT PCR. Delta Ct Method was calibrated with the mean of DCt at day zero (base line) as the first calibration, DCt of each sample at day zero (base line) as the second calibration and without calibration as the benchmarks for measuring the ratio. The relative expression ratio of osteogenic media to normal media was compared among the calibrations. The reliability and the variance of differences were statistical analysed by Bland-Altman and Pitman's Test.

Results: Calibration using the mean of DCt PDLSC on day zero showed no variance different with calibration using DCt of each PDLSC sample in normal media for PDLSC and GF (p= 0.055 and p=0.110 respectively). However, the low relative expression demonstrated a better reliability compared to high relative expression. The relative expression ratios of osteogenic media to normal media in all methods of calibration were reliable.

Conclusions: The results suggested that all the methods of calibration give the same results for the relative expression ratio.

RKWard: a Software Framework for Analysis of qPCR, dPCR, qIA and Melting Curve Data

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Several software solutions have been proposed for the analysis of qPCR, dPCR, quantitative isothermal amplification (qIA) or melting curve experiments (MCA). However, most of them are either tied to a specific task (e.g., qPCR data) or closed source software with little control over the analysis algorithms [1]. We started to build a comprehensive suite for the analysis of the various data types. Our software is based on the open source, cross-platform graphical user interface (GUI) and integrated development environment***RKWard*** [2]. We argue that our approach is useful for tracking numerical errors easy debugging and user-side code inspection. For rapid prototyping of plugin skeletons and XML structures for ***RKWard*** GUIs we used the ***rkwarddev*** package. Core algorithms are implemented as independent packages (***MBmca***, ***chipPCR***, ***RDML***, ***qpcR***, ***dpcR***) for statistical data analysis with the ***R*** language and have been published in peer-reviewed journals [1,3,4]. The RDML data exchange format will be part of the software to provide MIQE compliance. We started to implement methods for the analysis of qPCR, dPCR, qIA and MCA experiments with a strong focus on preprocessing. Our software is primarily targeted at users who develop novel devices or users who wish to analyze raw and unprocessed data from commercial systems (no "black box" approach). The complete analysis pipeline consisting of statistical procedures, raw data preprocessing, analysis, plots and report generation are implemented for increasingly demanded reproducible research [5]. The inherent open source nature of all components enables the design of highly customized analysis pipelines.

[1] Pabinger and Rödiger et al., *Biomolecular Detection and Quantification* (2014), **1/1**, 23-33. [2] Rödiger S et al., *Journal of Statistical Software (2012), 49* [3] Spiess AN et al.,*Clinical Chemistry (2015), preprint.* [4] Rödiger S et al., *The R Journal* (2013), **5/2**, 37. [5] Bustin S, *Biomolecular Detection and Quantification* (2015)

Advantages in Real-time PCR Quantification Using SOD and Cy0 Methods

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Background: the Cy0 method is a threshold-based method like Ct but with the key difference that the threshold value is dynamic and depends on amplification kinetic and possibly it should compensate for small variations among the samples to be compared. From a mathematical point of view, the Cy0 value has been defined as the intersection point between the abscissa axis and the tangent of the inflection point of the Richards curve obtained by the non-linear regression of raw data. This method combines the stability and reliability of a standard curve approach with a fitting procedure to overcome slight of PCR efficiency variations in real-time PCR nucleic acid quantification. Conversely, in the presence of marked initial amplification efficiency decreas, amplification runs shift to right and subsequentially the *Cy0* is impaired; hence, determination of initial amplification efficiency is essential to achieve a reliable quantification.

Aim: the aim of the present study is to propose an improvement in the *Cy0* method that is based on the use of kinetic parameters calculated in the curve inflection point to compensate for marked efficiency variations. Then, the enhanced *Cy0* method has been integrated with SOD analysis to quantify starting DNA quantity in the presence of different amplification kinetics.

Material and Methods**:** three experimental models have been used to test the precision and accuracy of the Cy0 corrected method: inhibition of primer extension and non-optimal primer annealing. Furthermore, to evaluate the output of the improved *Cy0* method in biological samples, we have quantified gene expression from skeletal muscle fine needle aspiration.

Results and Conclusions: the improved *Cy0* method significantly increased quantification accuracy without affecting precision in inhibition of primer extension and non-optimal primer annealing models. Then in order to evaluate the output of the improved *Cy0*method in biological samples, we have quantified gene expression from skeletal muscle fine needle aspiration. This technique has allowed us to recover only small quantities of biological materials, hence it is very difficult, if not impossible, to establish PCR efficiency using the standard curve method. Interestingly, the amplification system showing the lowest efficiency presented more outlier curves. The correction of the *Cy0* values using the efficiency parameters estimated in the inflection point have been performed only in the SOD positive runs.

In conclusion, the integration of the SOD with the improved *Cy0* method represents a simple but powerful approach to achieve reliable quantification even in the presence of a marked decrease in initial and variation in falling amplification efficiencies.

Poster "Late Arrivals" (Poster 74 - 77)

Automated Normalization and Pooling of DNA with the Labcyte Echo® Liquid Handler to enable Next Generation Sequencing.

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Next generation sequencers have evolved to sequence hundreds to thousands of samples in a single run by sequencing pools of DNA libraries that have been labeled with unique barcodes. The ability to efficiently pool and normalize such DNA libraries is now a critical requirement of any NGS library production process. In efforts to improve library product processes, many have learned that efficient utilization of sequencing capacity is realized only if the production and management processes associated with upstream library construction, normalization and pooling are also scalable and cost effective. In this study, we used the Echo liquid handler to normalize and pool the DNA fragments, quantified the DNA concentrations with the BMG LABTECH PHERAstar FS multimode high throughput reader, and validated the pooled fragments with the Agilent 2200 TapeStation. The ability of the Echo liquid handler to normalize and pool the DNA in a single step, coupled with the power and flexibility of the Labcyte Echo® applications software, provides a scalable, robust, high hroughput solution for an important upstream step in the NGS library production process.

High Throughput Miniaturized Quantitative PCR using the Echo® 525 Liquid Handler.

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Quantitative PCR (qPCR) is a prevalent tool spanning many phases of drug discovery. Advances in qPCR detection to enable 384- and 1536-well microplate formats have incentivized researchers to miniaturize qPCR assays as a means to offset the costs of increasing throughput. To significantly reduce qPCR volumes and maintain data quality, the liquid handling methods employed for such low-volume transfers must be precise and accurate. Tipless, touchless acoustic droplet ejection with the Echo liquid handler eliminates the cost of disposable tips or tip-wash cycles and simplifies assay setup by eliminating dilution steps. This study utilized the Echo 525 liquid handler to assemble low-volume qPCR assays at speeds that keep pace with high-throughput demands. 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%. The results confirm the advantages of using the Echo 525 liquid handler to miniaturize reaction volumes for high-throughput qPCR in both 384- and 1536-well formats.

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.

Applications of Next Generation Sequencing in Plant Breeding.

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Since 2005, when the first NGS sequencer was launched by Roche/454, NGS technologies have been undergoing rapid development. In 2014, the market leader Illumina introduces new updates for existing sequencers and launches new platforms. Promising technologies like Pacific Biosciences RS II and Oxford Nanopore MinIon come to the fore using innovative strategies.
For plant breeding, NGS applications have become indispensable. Restriction site associated DNA sequencing (RAD-Seq), can be used as an elegant tool to discover high quality markers for downstream applications like microarrays (Pegadaraju et al., 2013), or it can be used for the simultaneous screening of hundreds or thousands of markers in large numbers of plants and at low cost.
Sequence capture technologies have also become an important tool for plant breeding. By capturing chromosomal regions up to 200 Mbp corresponding to meta-quantitative trait loci (QTL) regions, it is possible to identify the genetic variation responsible for the different manifestations of the traits. Another application of sequence capture is the analysis of genetically modified organisms (GMOs). It is possible to prove successful transfections or show a complete crossing out of stable integrated genes (like antibiotic resistance genes) in reverse breeding. Using example data of particular projects, we will explain the technologies and typical applications thereof.

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