qPCR 2005 5 – 9th September 2005

Symposium & Exhibition & Workshop

2nd international qPCR Event, Technische Universität München,
Freising-Weihenstephan, Germany

Proceedings

qPCR 2005

2nd International qPCR Symposium
Industrial Exhibition
TATAA Application Workshop & qPCR Matrix Workshop

The whole story of quantitative PCR – from Tissue Preparation to BioInformatics

ISBN 3000166874

Editor: Michael W. Pfaffl

Physiology, Freising – Weihenstephan, Technical University Munich (TUM)
85354 Freising, Germany

http://qPCR2005.gene-quantification.info

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qPCR Event Overview

	Lecture hall 14	Lecture hall 15	Foyer & Seminar rooms 1 & 2	Practical room Nr. 2	Practical rooms Nr. 1, 3 and 4
	HS 14	**HS 15**	**Foyer, S1 & S2**	**P2**	**P1, P3 & P4**
Sun. 4th Sept. 2005			13:00 – 20:00 Built up		
			15:00 – 18:00 Arrival & Registration		
Mon. 5th Sept. 2005	10:00 – 10:20 **Welcome & Opening of the Symposium**		8:00 – 10:00 Arrival & Registration		
	10:20 – 11:00 Keynote lecture by **Russell Higuchi** ***"Pioneer in real-time PCR"***		10:00 – 19:00 **Industrial Exhibition**		
	11:00 – 17:00 **Pre-Analytical Steps**		17:00 **Refreshments in the Industrial Exhibition**		17:00 – 18:30 **Organization of Matrix Workshop Sessions at Reg. Desk**
	18:30 – 20:00 Poster – Session				
	20:00 – 24:00 Poster – Party Salonorchester Karl Edelmann is presenting a variety of international music Poster Party is sponsored by **Roche Applied Science & Eppendorf**				
Tue. 6th Sept. 2005	8:00 – 10:10 **New Applications: Single Cells**	8:00 – 10:10 **Normalization**	8:00 – 18:00 **Industrial Exhibition**		
	10:40 – 12:30 **New Applications: New Methods**	10:40 – 12:50 **Optimization – part 1**			
	13:30 – 16:00 **New Applications: Multiplexing**	13:50 – 15:20 **Optimization – part 2**			
	16:30 – 18:30 **New Applications: Mixed Session**	15:50 – 18:00 **Standardization**			
	19:00 – 24:00 Symposium Gala Dinner Location: Lindenkeller, Pasta & More, Freising Baverian Buffet, Mediterranean Buffet, Asian Buffet, Modern Crossover Buffet, Music & Dancing				
Wed. 7th Sept. 2005	8:00 – 12:10 **BioInformatics**	8:00 – 12:10 **GMO Analytics & Food Hygiene**	8:00 – 13:00 **Industrial Exhibition**		
	12:10 – 12:20 **Closing of the Symposium**			13:00 – 18:00 **TATAA qPCR Application Workshop**	13:00 – 18:30 **qPCR Matrix Workshop**
Thu. 8th Sept. 2005				9.00 - 17:00 **TATAA qPCR Application Workshop**	8:30 – 18:30 **qPCR Matrix Workshop**
Fri. 9th Sept. 2005				9:00 - 17:00 **TATAA qPCR Application Workshop**	8:30 – 16:00 **qPCR Matrix Workshop**

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Invitation

Freising, August 2005

Dear colleagues,
dear researchers,
dear company representatives,

on behalf of the Organisation Committee and the Scientific Board it is great pleasure to invite you to the qPCR 2005 Event, the 2nd International qPCR Symposium & Application Workshop to be held September 5-9, 2005, at the Center of Life Science in Freising Weihenstephan, Technische Universität München (Germany). The great international interest in qPCR 2004 - 1st International qPCR Symposium & Application Workshop from 44 countries and 32 international companies in the qPCR Industrial Exhibition led us to the decision to repeat it in 2005. The event will focus on all aspects of qPCR technology and its applications in research and diagnostics. Leading academic researchers and industrial contributors in the field will be participate in the symposium, which will be an arena for fruitful discussions between researchers of different backgrounds. The Symposium, the Industrial Exhibition and two types of associated Workshops offer an overview of the present knowledge and future developments in qPCR technology and its wide applications.

The subtitle of the qPCR 2005 events is: **The whole story of quantitative PCR – from Tissue Preparation to BioInformatics**

The event is divided in

1. **qPCR Symposium** from 5 – 7th September,
2. **qPCR Industrial Exhibition** from 5 – 7th September
3. qPCR Workshops from 7 – 9th September: **TATAA Application Workshop** and **qPCR Matrix Workshop**

The location is the central lecture hall and the foyer at TUM (Technical University Munich) in Freising Weihenstephan. We are part of the biggest Biotech Cluster in Germany and Europe, located close to the Munich airport, and in the heart of Bavaria.

The scientific organization team:

Scientific board:

Stephen Bustin	Prof. of Molecular Science QM, School of Medicine, London, UK
Mikael Kubista	Prof. of Biotechnology, MultiD Analyses AB, Gothenburg, Sweden
Vladimir Benes	EMBL, Genomics Core Facility, Heidelberg, Germany
Neven Zoric	Coordinator of TATAA Biocenter, Sweden (coordinator TATAA Application Workshop)
Heinrich H.D. Meyer	Prof. of Physiology, Weihenstephan, Germany
Michael W. Pfaffl	Reader in Physiology, Weihenstephan, Germany scientific coordinator of the Symposium and the Matrix Workshop qPCR2005@wzw.tum.de

Special guest:

Russell G. Higuchi	Pioneer in real-time PCR, Associate Director of the Human Genetics Department, Roche Molecular Systems, Alemada, CA, USA
Event organization:	Ulrich Wild, TUM-Tech GmbH Munich ulrich.wild@tumtech.de

I am looking forward meeting you in September at the Symposium in Freising-Weihenstephan.

Michael Pfaffl
Sympoium Chair

Press release

The whole story of quantitative PCR – from Tissue Preparation to BioInformatics

The qPCR 2005 Event is organized jointly by Chair of Physiology, Technical University of Munich (TUM), and TUMTech-GmbH, Munich, Germany

http://qpcr2005.gene-quantification.info

The Physiology Weihenstephan at the Technical University of Munich with support from TUM-Tech GmbH have taken the initiative to organize the qPCR 2005 Event. From 5th – 9th September 2005 more than 350 scientists from 45 countries will exchange ideas, share experiences, and discuss the exciting future of the perhaps most powerful analytical technology ever developed in the life sciences area – **the quantitative real-time polymerase-chain-reaction (qPCR).**
Parallel to the symposium, an industrial exhibition takes place where 26 worldwide leading biotechnology companies will present there newest PCR technologies, e.g. real-time PCR cyclers, nucleic acid extraction robots, DNA and RNA detection and amplification chemistry, as well as software applications.

qPCR, is an improved technology based on PCR that was awarded 1993 years Nobel price in Chemistry. Using qPCR the amount of target nucleic acid in a complex sample can be determined with high precision, absolute correctness, excellent specificity and the ultimate sensitivity of detecting even single molecule. The technique has revolutionized all molecular sciences and diagnostic applications. Hospital laboratory tests that used to take hours, sometime days to perform, and required the handling of hazardous chemicals, can today be made quickly in fully automated systems. Conference presentations will show that in near future, using improved instrumentation, sample preparation, extraction, and qPCR application will take a minimum of time and the test results will be delivered while waiting. The combination with reverse transcription enables determination of mRNA and widely opens the window for *"Transcriptomics"* – the first step of gene expression and functional genomics.

The subtitle of the conference is: **The whole story of quantitative PCR – from Tissue Preparation to BioInformatics**
In the meeting the most critical steps in real-time qPCR will be discussed in detail. More than 50 invited international speakers from all over the world present their knowledge around qPCR. There is almost no field in life sciences not open to many qPCR applications for nucleic acid analysis.
Presentations at the conference are divides in specialized sessions, starting with pre-analytical steps, e.g. improving nucleic acid extraction and stabilizing. In the biggest section, called new applications, the focus is on single-cell and single-molecule qPCR, development of new detection and application methods, using various multiplexed dyes in qPCR, and verification of array results via qRT-PCR. Parallel session will show how sampling and amplification performance can be increased by optimization and standardization procedures, e.g. in the tropics or in cancer diagnostics. Totally new normalization techniques will be presented on the basis of ALU repeats or total DNA. Further developments of qPCR technology focus on miniaturization, higher throughput, cost efficacy, validity, and flexibility.
Accurate GMO quantification in food samples, identification and quantification of pathogens in plant/ animals are further key topics. Goal is to develop fast, sensitive, and highly reproducible GMO real-time amplification methods.
A highlight of the symposium are the qPCR BioInformatics. New algorithms and quantification software will be presented to improve the real-time data acquisition, amplification efficiency calculation, detection of outliers, normalization of gene expression, and multi-factorial data management of real-time results (e.g. qBASE, REST, DATAN, KOD).

In connection with the symposium two independent practical qPCR workshops take place from 7th – 9th Sept.: TATAA Application Workshop and the qPCR Matrix Workshop. Application workshop is hosted by the TATAA Biocenter (http://www.tataa.com), which is the leading qPCR service provider in Europe. TATAA is associated with Chalmers University of Technology and the University of Göteborg, Sweden. The 11 sessions of the Matrix Workshop will present a broad variety of new cyclers, hot technologies and detection chemistries. The Matrix Workshop sessions are hosted by the R&D scientists from biotechnology companies and by international renown molecular biologists.

The Physiology Weihenstephan at the Center of Life and Food Sciences at Technical University of Munich, chaired by Prof. Heinrich H. D. Meyer, is a leading authority in the molecular physiology of mammalian species. Cutting edge biochemical and molecular biology techniques are established for basic and applied research on the regulation of reproduction, lactation, immunology, and growth. Both traditional endocrinology and paracrine regulations are studied in numerous tissues. Michael W. Pfaffl is developing qRT-PCR methods, software algorithms, and tools for quantitative gene expression analysis. http://www.gene-quantification.info

For more information about the qPCR 2005 Event see http://qpcr2005.gene-quantification.info
or contact Dr. Michael W. Pfaffl qPCR2005@wzw.tum.de or Dr. Ulrich Wild ulrich.wild@tumtech.de

Agenda qPCR 2005

Sunday 4th September 2005

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

15:00 – 18:00 Arrival & Registration

Monday 5th September 2005

Welcome & Opening of the Symposium
Lecture hall HS 14

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

08:00 – 10:00 Arrival & Registration

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

10:00 Welcome & Opening of the Symposium.
Michael W. Pfaffl & Neven Zoric
Scientific coordination of the qPCR 2005 Symposium & TATAA Application Workshop

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

10:20 **Keynote lecture:**
Real-time PCR, a personal perspective.
Russell Higuchi, *"Pioneer in real-time PCR"*
Associate Director of the Human Genetics Department, Roche Molecular Systems, Almeda, CA, USA

Session: Pre-Analytical Steps
Chair: V. Benes
Lecture hall HS 14

11:00 Session introduction by V. Benes

11:10 mRNA quantification from archival cancer samples.
Stephen A Bustin, Rebecca Hands, Sina Dorudi, Institute of Cell and Molecular Science, Queen Mary's School of Medicine and Dentistry, University of London

11:40 Nucleic acid isolation for diagnostic testing using Bayer´s magnetic particles.
Guido Hennig, Bayer HealthCare AG, Diagnostics Research Germany, Leverkusen

12:10 Robust molecular profiling from RNA derived of archival tissue.
Janine Antonov, Departement of Clinical Research, University of Bern, Switzerland.

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

13:40 Nucleic Acid Stabilization in Cultured Cell and Tissue Lysates for QPCR Gene Expression Analysis.
L. Scott Basehore, Sr. Research Associate, Stratagene Research & Development Department

14:10 Standardization of RNA Quality Assessment using the RNA Integrity Number (RIN) and the 2100 bioanalyzer.
Marc Valer, Agilent Technologies, Waldbronn, Germany
LIVE presentation of Bioanalyzer 2100

15:00 – 15:30 **Coffee break**

15:30 Influence of RNA matrix effect on qRT-PCR results – an overview.
Michael W. Pfaffl, Simone Fleige, Physiology, Center of Life Science, Weihenstephan, Technical University of Munich, Germany

16:00 Use of standardized mixtures of internal standards in RT-PCR to generate validated biomarkers and to develop standardized transcript abundance reference databases.
James Willey[1], Elizabeth Peters [2], Charles Knight[1], Erin Crawford, [1], Bradley Austermiller[1], Terry Osborn[2]; 1: Medical University of Ohio, Toledo, Ohio, United States. 2: Gene Express, Inc., Toledo, Ohio, United States.

16:30 Optimization of reverse transcription for two-step QRT-PCR: A comparison of RT priming methods and the addition of a new enhancer for efficient removal of double-stranded DNA contamination.
Ian Kavanagh[1], Stephanie Noel[1], Chatu Rajapakshe[1], Gerwyn Jones[1], Nicky Quispe[1], Simon Baker(1, 2), Meg Martel[1], 1: ABgene, Epsom, United Kingdom. 2: Birkbeck, University of London, United Kingdom.

17:00 – 18:30 **Refreshments in the Industrial Exhibition**
Get-together with the Companies

18:30 – 20:00 **Poster - Session**

20:00 – 24:00 **Poster – Party**
welcome by Prof. Heinrich H.D. Meyer
Salonorchester Karl Edelmann is presenting a variety of international music

Poster Party is sponsored by

Roche eppendorf

Tuesday 6th September 2005

Session: New Application – part 1: single cells
Chair: M. Kubista
Lecture hall HS 14

08:00 Session introduction by M. Kubista

08:10 Gene expression profiling in single cells.
Anders Ståhlberg (1), Martin Bengtsson (1,2), Patrik Rorsman(2,3) and Mikael Kubista (1)
1: Department of Chemistry & Bioscience / Molecular Biotechnology, Chalmers University of Technology and TATAA Biocenter, Sweden. 2: Department of Experimental Medical Science, Lund University, Sweden. 3: The Oxford Centre for Diabetes, Endocrinology and Metabolism, The Churchill Hospital, Oxford, England.

08:40 Quantitative single-cell RT-PCR and calcium imaging in acute brain slices.
Robert Blum, Guylaine M. Durand, Nima Marandi, Simone D. Herberger, Arthur Konnerth, Ludwig-Maximilians-Universität, Germany.

09:10 Amplification based assays in nanoliter volume range.
Andreas Dahl[1], Marc Sultan[1], Regine Schwartz[1], Matthias Lange[1], Alexander Jung[2], Michael Steinwand[2], Kenneth Livak[2], Hans Lehrach[1] and Lajos Nyarsik[1], (1) Max Planck Institute for Human Genetics, Deutschland (2) Applied Biosystems.

09:40 Forensic and single-molecule assays of mitochondrial DNA using LATE-PCR.
Arthur Reis, Lawrence J. Wangh, Brandeis University, Boston, MA, USA

10:10 – 10:40 **Coffee break**

Session: New Application - part 2: new methods
Chair: R. Higuchi
Lecture hall HS 14

10:40 Rapid Development of RT-PCR Assays for RNAi Experiments Using Pre-designed LNA-probe Libraries.
Michael Boutros, Boveri-Group Signaling and Functional Genomics, DKFZ, Heidelberg, Germany.

11:10 A Multiplex Branched DNA Assay for Parallel Quantitative Gene Expression Profiling.
Michael Flagella*, Son Bui*, Zhi Zheng, Cung Tuong Nguyen, Aiguo Zhang, Larry Pastor, Yunqing Ma, Wen Yang, Kim Crawford, Gary K. McMaster, Frank Witney and Yuling Luo, Genospectra Inc., United States.

11:40 TripleHYB: A novel detection format for real-time PCR.
Anne-Katrin Rost [1], Natalia Malchowa [2], Awad A. Osman [1], Thomas Köhler [1], 1: AJ Roboscreen GmbH, Delitzscher Strasse 135, D-04129 Leipzig, Germany. 2: Department of Microbiology, Faculty of Biotechnology, Jagiellonian University, Gronostajowa 7, 30-387 Cracow, Poland.

12:00 Correlation of microarray and quantitative real-time PCR results.
Elisa Wurmbach, Mount Sinai School of Medicine, United States.

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

Session: New Application - part 3: multiplexing
Chair: S. Bustin
Lecture hall HS 14

13:30 The best of both worlds - New dyes for qPCR for use in combination with probes.
Neven Zoric, Coordinator of the TATAA Biocenter, Göteborg, Sweden.

14:00 qPCR pitfalls - primer and probe design / fluorophore quencher combinations.
Clémence Beslin, Eurogentec, Belgium.

14:30 Going MULTI – how to easily achieve high multiplexing in real-time PCR.
Andreas Missel, Associate Director R&D, QIAGEN GmbH, Hilden

15:00 Plexor™ Real-Time Quantitative PCR Systems: Multiplexed assays made easy.
Kyle Hooper, Promega Corporation, Woods Hollow Road, Madison, WI, USA

15:30 Two-color multiplex assay for the identification of Orthopoxes viruses with Real-Time LUX PCR.
Mohamed Aitichou [1], Sandrine Javorschi-Miller [2], Sofi Ibrahim [1], Mark Andersen [2], 1: Virology Division, United States Army Medical Research Institute of Infectious Diseases, United States. 2: Invitrogen, United States.

16:00 – 16:30 **Coffee break**

Session: New Application - part 4: mixed session
Chair: T. Bar
Lecture hall HS 14

16:30 ChIP studies on a HOX gene regulated by Polycomb group and trithorax group proteins.
Bernadett Papp, EMBL, Germany.

17:00 The Ups and Downs of Gene Regulation: Validating siRNA Gene Expression Disruption with RT-qPCR.
Hilary Katherine Srere, Bio-Rad Laboratories, United States.

17:30 Real-time immuno-PCR for quantification of proteins.
Kristina Lind, Mikael Kubista; Department of Chemistry and Bioscience, Chalmers University,

18:00 microRNA expression profiles from Real-time PCR classify ES and differentiated cells
Simone Guenther [1], Adam Broomer [2], Dana Ridzon [2], Kai Lao[2], Karl Guegler[2], William Strauss[3]; 1: Applied Biosystems, Darmstadt, Germany. 2: Applied Biosystems, Foster City,USA. 3: University of Colorado, Boulder, USA.

19:00 – 24:00 **Symposium Gala Dinner**
Location: Lindenkeller, Pasta & More, Freising
- Bavarian Buffet
- Mediterranean Buffet
- Asian Buffet
- Modern Crossover Buffet
- Music and Dancing

Tuesday 6th September 2005

Session: Normalization
Chair: N. Zoric
Lecture hall HS 15

08:00 Session introduction by N. Zoric

08:10 Normalization of gene expression: state of the art and preview on a new strategy using expressed Alu repeats.
Jo Vandesompele, Center for Medical Genetics Ghent, Ghent University Hospital, Ghent, Belgium

08:40 Normalisation of mRNA levels against total DNA content.
Shu-Rui Li [1], Doug Storts [2], Becky Hands [1], Benjamin Krenke [2], Ethan Strauss [2], William Ogunkolade [1], Stephen Andrew Bustin [1], 1: Queen Mary University of London, United Kingdom. 2: Promega Corporation, USA, Institute of Cell and Molecular Science, Queen Mary's School of Medicine and Dentistry, University of London

09:10 Normalization genes for heart failure myocardium in mice, rats and humans.
Trond Brattelid (2,3), Lisbeth Winer (1), Ole M. Sejersted (1,3) and Kristin B. Andersson (1,3)
1: Institute for Experimental Medical Research, Ullevaal University Hospital, University of Oslo. 2: Department of Pharmacology, University of Oslo. 3: Center for Heart Failure Research, Faculty of Medicine, University of Oslo.

09:40 Early mouse development and mammalian embryonic stem cells: a qRT-PCR story.
Erik Willems[1], Caroline Kemp[1], Ileana Mateizel[2], Karen Sermon[2] and Luc Leyns[1], 1. Lab for Cell Genetics, Vrije Universiteit Brussel, Brussels, Belgium. 2: Research Centre for Reproduction and Genetics, Vrije Universiteit Brussel, Brussels, Belgium.

10:10 – 10:40 **Coffee break**

Session: Optimisation – part 1
Chair: B. Rutledge
Lecture hall HS 15

10:40 Session introduction by b. Rutledge

10:50 Design and optimization of Taqman and SYBR Green I real-time qPCR assays.
Greg Shipley, Director, Quantitative Genomics Core, Laboratory, The University of Texas Health Science Centre-Houston, USA

11:20 Comparison of MMP gene expression analysis by capillary and "realplex" real-time PCR.
Raimund Kinne, Experimentelle Rheumatologie, Klinikum der Friedrich-Schiller-Universität Jena, Germany

11:50 Finding the needle in the haystack - LNA bases enhance SNP detection dramatically.
Olfert Landt, TIB MOLBIOL Syntheselabor GmbH Eresburgstraße, Berlin, Germany

12:20 Infectious disease diagnostic research in Africa; the role of real time PCR.
Jim Huggett, Centre for Infectious Diseases & International Health, University College London, UK

12:50 – 13:50 **Lunch in the student cafeteria**

Session: Optimization – part 2
Chair: H.H.D. Meyer
Lecture hall HS 15

13:50 Relative real time PCR for gene expression measurement in breast cancer biopsies.
A.Larionov[1], S.White[1], D.B.Evans[2], A.Krause[2], M.J.Dixon[1], W.R.Miller[1]; 1: Breast Research Group, Western General Hospital, Edinburgh, UK. 2: Novartis Pharma AG, Basel, Switzerland.

14:20 The fitness of a football team: High Resolution Melts for the determination of genotypes.
Valin Reja[1], Brant Bassam[1] and Thomas Kaiser[2]; 1: Corbett Research, Mortlake, NSW, Australia. 2: Corbett Research UK Limited, Cambridge Science Park, Milton, Cambridge, UK.

14:50 Validation of fast PCR protocols with the Eppendorf Mastercycler ep realplex.
Cynthia Potter, Eppendorf UK Limited, Vision Park, Chivers Way, Histon, Cambridge, UK

15:20 – 15:50 **Coffee break**

Session: Standardization
Chair: G. Shipley
Lecture hall HS 15

15:50 Session introduction by G. Shipley

16:00 A Comparison of Real-Time RT-PCR Technique, Chemistries and Instrumentation in Laboratories Utilizing the Same Assay.
Pamela Scott Adams, Director, Molecular Biology Core Facility, Trudeau Institute, Saranac Lake, NY, USA

16:30 Accurate Gene Expression Analysis with High Flexibility: Concepts and Developments.
Oliver Geulen, Roche Applied Science, Mannheim, Germany

17:00 The Data Comparability Challenge - Standards and Best Practices.
Morten T. Andersen, Bio-Molecular Innovation, LGC, Teddington, Middlesex, UK

17:30 Putting the "quantity " into quantitative PCR: A simplified approach to the establishment and application of quantitative scale.
Bob Rutledge, Natural Resources Canada, Canadian Forest Service, Laurentian Forestry Centre, Sainte-Foy, Quebec, Canada

18:00 Assay standardisation using universal internal controls and lyophilized reagent beads.
Andreas Eckelt, Cepheid SA, Deutschland.

19:00 – 24:00 **Symposium Gala Dinner**
Location: Lindenkeller, Pasta & More, Freising
- Bavarian Buffet
- Mediterranean Buffet
- Asian Buffet
- Modern Crossover Buffet
- Music and Dancing

Wednesday 7th September 2005

Session: BioInformatics
Chair: M. W. Pfaffl
Lecture hall HS 14

08:00 Session introduction by M. W. Pfaffl

08:10 From Sequences to Synthesis: Optimal Amplification through Careful Oligonucleotide Selection.
Ben Sowers, Research Associate, Biosearch Technologies

08:40 Estimation of sample specific efficiency – methods and applications.
Tzachi Bar, Department of Chemistry and Biosciences Chalmers University of Technology, Göteborg, Sweden; Ales Tichopad, LabonNet, Kirchheim bei München, Munich, Germany

09:10 Amplification efficiency dynamics and its implications: Developing a kinetic-based approach for quantitative analysis.
Bob Rutledge, Natural Resources Canada, Canadian Forest Service, Laurentian Forestry Centre, Sainte-Foy, Quebec, Canada

09:40 qBase: relative quantification software for management and automated analysis.
Jan Hellemans, Center for Medical Genetics Ghent, Ghent University Hospital, Ghent, Belgium

10:10 – 10:40 **Coffee break**

10:40 Early Phase Fluorescence Fitting of real-ime PCR reaction.
Hervé Rhinn, Laboratoire de Pharmacologiqe Chimique et Génétique, France.

11:10 Classification of real-time PCR data.
Mikael Kubista, Professor of Biotechnology, MultiD Analyses AB, Göteborg, Sweden

11:40 The real-time PCR primer and probe database RTPrimerDB: a major update.
Filip Pattyn, Piet Robbrecht, Jelle Verspurten, Anne De Paepe, Frank Speleman, Jo Vandesompele, Center for Medical Genetics Ghent (CMGG), Ghent University Hospital, Ghent, Belgium.

12:10 Closing of the Symposium in HS 14
Michael W. Pfaffl

Wednesday 7th September 2005

Session: GMO Analytics & Food Hygiene
Chair: C. Albrecht
Lecture hall HS 15

08:00 Session introduction by C. Albrecht

08:10 **Keynote lecture:**
Uncertainties and certainties in GMO analytics using qPCR.
Philipp Hübner, Kantonales Laboratorium Basel-Stadt, Abteilungsleiter Lebensmittel, Basel, Switzerland.

08:50 Accurate GMO quantification in food samples.
Dörte Wulff, Research and Development, Eurofins Genescan / GeneScan Analytics GmbH, Freiburg, Germany

09:20 The USDA/GIPSA Proficiency Program: A Summary of Participants Capabilities for Detecting and Quantifying Transgenic Events in Corn and Soybeans.
Ron Jenkins, USDA/GIPSA, USA

09:50 - 10:30 **Coffee break**

10:30 Application of synthetic DNA-standards for the quantitative screening of different genetically modified rapeseed lines via real-time PCR.
Francisco Moreano, Bayerisches Landesamt für Gesundheit und Lebensmittelsicherheit (LGL), Infektologie / Molekularbiologie, German

11:00 Cloned plasmid DNA molecules as a tool for GMO analysis.
Isabel Taverniers, Marc De Loose
Department of Plant Genetics and Breeding, DvP-CLO, Melle, Belgium.

11:30 Detection of Food Pathogens using the Smart Cycler II.
Martina Fricker, Dep. of Bioscience, Technical University of Munich, Freising, Germany

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Closing of the Symposium
Lecture hall HS 14

12:10 – 12:20 Closing of the Symposium.
Michael W. Pfaffl

12:20 – 13:00 **Lunch in the student cafeteria**

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www.gene-quantification.info

Oral presentations:

Welcome & Opening

Time: 5th Sep 2005, 10:00:00 - 5th Sep 2005, 11:00:00
Session Chair: Dr. Michael W. Pfaffl
Location: lecture hall 14 (HS 14)

Keynote Lecture:

Real-time PCR, a personal perspective.

Russell Gene Higuchi (russell.higuchi@roche.com)
Roche Molecular System, United States.

Early days and applications of real-time PCR will be reviewed. Review of inherent sensitivity constraints on quantification by hybridization (i.e., microarrays) compared with real-time PCR. Use of real-time PCR in genome-wide studies of association of disease to DNA polymorphisms.

Session

Pre-Analytical Steps

Time: 5th Sep 2005, 11:00:00 - 5th Sep 2005, 17:00:00
Session Chair: Dr Vladimir Benes
Location: lecture hall 14 (HS 14)

mRNA quantification from archival cancer samples.

Rebecca Hands, Sina Dorudi, Stephen Andrew Bustin (s.a.bustin@qmul.ac.uk)
Queen Mary University of London, United Kingdom.

Formalin-fixed, paraffin-embedded tissue provides a vast source of morphologically defined biopsies derived from normal and diseased tissue for which extensive clinical data are available. However, poor accessibility and lack of integrity of cellular mRNA has made its reliable quantification difficult. Recent improvements to reagents, combined with developments in laser microdissection technology now make it possible to extract and amplify reliably even low copy number mRNA targets. The aim of this talk is to discuss some of the issues relevant to anyone interested in using archival material for expression analysis.

Nucleic acid isolation for diagnostic testing using Bayer´s magnetic particles.

Guido Hennig (guido.hennig@bayerhealthcare.com)
BayerHealthCare AG, Diagnostics Research Germany.

Bayer HealthCare's Business Group Diagnostics develops nucleic acid detection platforms for infectious disease, oncology and pharmacogenomic testing. The efficient purification of nucleic acids from biological samples with regards to interference free eluate and high recovery is a key step for subsequent analysis. The required performance combined with robustness, throughput, process time, system flexibility, and adaptability to automation could be achieved best by employing magnetic bead technology.
Bayer Diagnostics Research has developed new silica-coated paramagnetic particles. These magnetic particles simplify and improve automated nucleic acid isolation. The particle properties are conferred by a nanolayer of silica which binds nucleic acids selectively, a very small particle size contributing to superior suspension behavior, a homogenous size distribution and strong magnetization.
Using these particles it is possible to establish automated protocols for isolation of viruses from the infectious disease panel (HCV, HIV and HBV) using industry-standard liquid handling platforms. In addition, assays were developed for sensitive detection of the respective viruses (10 copies/reaction). The presented model system for automated HCV isolation and detection allows flexible purification of up to 96 samples in less than 2.5 hours with a very good linearity, reproducibility, specificity and sensitivity.
Furthermore these new Bayer magnetic particles are flexible in its biological applications and also allow the isolation of genomic DNA from blood for pharmacogenomic testing and DNA and RNA from tumor tissues. Examples for these applications will be presented and discussed.

Robust molecular profiling from RNA derived of archival tissue.

Janine Antonov (janine.antonov@dkf.unibe.ch)
1: . Departement of Clinical Research, University of Bern, Switzerland.
2: . Pathologie Länggasse, Bern, Switzerland.

Conventional classification of breast cancer is based on clinical and histological parameters, supplemented by immunohistochemical analyses for some selected genes. Over the years, large batteries of samples have been collected which are stored as formalin-fixed, paraffin-embedded (FFPE) material in many pathological institutes. However, classification based on these parameters is still unsatisfactory and recently developed, molecular approaches which are based on gene expression measurements for multiple genes were developed. They allow a more detailed characterization of individual tumors and they have a great impact on further treatment modalities. Unfortunately, RNA which can be isolated from FFPE material is considerably degraded and expression measurements from such RNA are difficult and often impossible at least with standard protocols and reagents.
We improved existing protocols for RNA isolation and cDNA synthesis, and we optimized primers and probes for quantitative PCR. Every gene-specific assay is subjected to a rigorous control and validation procedure and only assays which are sufficiently independent on the extent of RNA fragmentation can be used for quantitative measurements of FFPE-derived RNA. Similarly, each RNA which is prepared from FFPE material is subjected to several control experiments. The proposed control and validation experiments result in a normalization factor for each RNA sample. This factor integrates the critical parameters which affect expression measurements by quantitative PCR (e.g. RNA fragmentation, efficiency of qPCR).
We performed a series of test experiments with tumor samples for which high quality RNA (snap frozen, RNAlater preserved material) and poor quality RNA (FFPE-derived material) of the same tumors were used and we observed highly reproducible profiles in both series of samples. Data will be presented on the procedures, primer design, normalization and quality controls which are necessary and sufficient to generate highly reproducible expression profiles from RNA derived of FFPE material.

Nucleic Acid Stabilization in Cultured Cell and Tissue Lysates for QPCR Gene Expression Analysis.

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A novel buffer is described for lysis of cells and stabilization of released nucleic acids. Lysed cells can be stored for three months or longer at -20°C before QPCR analysis is performed. RNA in stored cell lysates shows little or no degradation by Agilent Bioanalyzer analysis. RNA and DNA can be amplified from cultured cell and tissue lysates without the need for purification. The lysate contains the entire nucleic acid complement, allowing use of single copy DNA markers to normalize input cell mass and accurately measure gene expression between samples by QPCR.

Standardization of RNA Quality Assessment using the RNA Integrity Number (RIN) and the 2100 bioanalyzer.

Marc Valer[1], Hans Brunnert [1], Rainer Wittig [2], Andreas Schroeder [1], Thomas Ragg [3], Juergen Schneider [1] (marc_valer@agilent.com)
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2: German Cancer Research Center (DKFZ).
3: Quantiom Bioinformatics.

Good RNA quality assessment is considered one of the most critical elements to obtain meaningful gene expression data via microarray or real-time PCR experiments. Advances in microfluidic technology have improved RNA quality measurements by allowing a more detailed look at patterns of RNA degradation via the use of electrophoretic traces. However, the interpretation of such electropherograms still requires a certain level of experience and can vary from one researcher to the next. The RNA integrity number(RIN) algorithm is introduced to assign a user-independent integrity number to each RNA sample. The RIN has been developed using neural networks by "teaching" this algorithm with a large number of RNA integrity data. The RIN score, based on a quality numbering system from 1-10 (in ascending quality), facilitates the classification of RNA samples to be used in the context of the gene expression workflow. In order to correlate microarray results with RIN and other alternative RNA quality measures, samples degraded for different times, where hybridized on microarray chips together or in parallel experiments. The results clearly show an important influence of the RNA sample integrity state as determined by the RIN towards the accuracy and noise levels in the array experiments.

Influence of RNA matrix effect on qRT-PCR results - an overview.

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For successful quantitative mRNA analytics it is important to use intact RNA. Tissue sampling, storage, and RNA extraction are the most sensitive and variable pre-analytical steps for quantitative downstream applications. The determination of RNA quantity and quality are critical first steps in obtaining meaningful gene expression data. The verification of the RNA integrity before use in different applications, e.g. quantitative RT-PCR (qRT-PCR) and microarray experiments, permits to compare experiments and classify the significance of results.
In this approach we optimised and standardized the total RNA extraction procedures to get integer and more reproducible total RNA quantity and quality. Total RNA was investigated with various methods: Photometer (Eppendorf), Bioanalyzer 2100 (Agilent Technologies) and Experion (Bio-Rad). The intention was to develop an evaluation system for the RNA integrity and the later influence on the qRT-PCR results in real-time applications. In this system a certain RIN threshold level should present where qRT-PCR breaks down leads or lead to reliable results.
Therefore the RNA Integrity Number (RIN) values, and 18S/28S ratios of different bovine tissues were correlated with the crossing points (CP) of qRT-PCR experiments. Following bovine tissues were analysed: WBC, liver, muscle, ileum, jejunum, reticulum, lymph nodes, colon, lung, corpus luteum, caecum, spleen, abomasums and various cell cultures. In the quantitative real-time RT-PCR primers for high-, intermediate- and low abundant genes were used: 18S, 28S, ß-Actin and Interleukin-1. Besides we compared the effects of different RNA degradation methods (enzymatic and physical degradation) and different qRT-PCR product lengths.
The correlation and regression between the CP and RIN showed, that increasing RIN numbers lead to a CP shift towards lower cycle numbers, independent of tissue and genes analysed. This effect was higher distinct in the physical than in the enzymatic degraded tissues. The correlation between the PCR efficiency from the qRT-PCR measurements and the RIN for all tested genes and tissues showed that the PCR efficiency in general not affected by the RIN. It was not possible to develop an evaluation system and a certain threshold RIN value for PCR products up to 200 bp. Both, bad (RIN = 1-3) and good RNA samples (RIN = 8-10), led to reliable PCR product. Only for longer PCR products over 400 bp a significant negative influence and a threshold of RIN = 5 could be shown. Using relative quantification and calculating delta-CP values to an internal control (housekeeping gene like beta-actin) the negative RIN influence on shifted delta-CP disappeared, and resulted in a constant CP level, independent on RNA integrity.

Use of standardized mixtures of internal standards in RT-PCR to generate validated biomarkers and to develop standardized transcript abundance reference databases.

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With Standardized RT (StaRT)-PCR™, a cDNA internal standard within a standardized mixture of internal standards (SMIS™) is included in PCR amplification of cDNA from each gene. This approach controls for all known sources of variation, including inter-sample variation in gene-specific inhibitors of PCR, variation in reverse transcription (RT) efficiency, and variation in loading. Yield of cDNA from RT may vary from 5% to 80% among multiple RTs of the same RNA. However, as long as the same priming method is used (e.g. oligo dT, sequence specific, or random primer), the relative representation of one transcript to another in the cDNA produced is highly reproducible. Thus, controlling for variation loading of cDNA into the PCR reaction controls for variation in RT efficiency, and inclusion of RNA standards in the RT is unnecessary. Data will be presented to support this assertion. Further, data will be presented from multi-institutional blinded studies supporting the claims that with StaRT-PCR™, primers and internal standard for each gene are quality-controlled to ensure a lower detection limit of less than 10 molecules, a linear dynamic range of over 7 orders of magnitude, a signal-to-analyte response of 100%, transcript specificity, and high reproducibility across experiments and laboratories (the average CV being less than 10%). Due to these performance characteristics, StaRT-PCR™ is ideal for generating data that meet the criteria established by US regulatory agencies (FDA and CDC) for inclusion in validated biomarkers. Data will be presented from clinical trials demonstrating the clinical utility of StaRT-PCR™ based biomarkers for improved diagnosis of bronchogenic carcinoma. The Standardized Expression Measurement (SEM™) Center uses Standardized RT (StaRT)-PCR™ to generate a reference database of transcript abundance (TA) data from normal and abnormal tissues necessary for clinical implementation of TA-based biomarkers. The clinical utility of a reference database generated from analysis of 19 genes in normal peripheral blood leukocytes from 15 normal subjects will be presented.

Optimisation of reverse transcription for two-step QRT-PCR: A comparison of RT priming methods and the addition of a new enhancer for efficient removal of double-stranded DNA contamination.

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QRT-PCR has a wide range of applications in many biological disciplines, including the measurement of gene expression. The increase in the use of this method has required research to improve the efficiency of a reaction, its reproducibility and the total time of the procedure. There are many choices a user must make in order to establish a viable procedure and factors such as RNA quality, primer design or choice of detection chemistry can significantly affect the sensitivity and efficiency of a reaction. Similarly, a choice must also be made over the type of primer to be used for initiating reverse transcription (RT) of RNA to produce cDNA. Currently, the main priming strategies employ either random sequences of nucleotides (typically 6-10 nucleotides in length) or oligo-dT (which will prime from the poly-adenosine tail present on the majority of eukaryotic transcripts). More recently an anchored oligo-dT has been employed, that primes at the mRNA/poly-adenosine tail junction, permitting optimal transcription of the gene-encoding region of the mRNA sequence. Here, we compare the effect that a variety of different RT priming methods can have on the sensitivity of QPCR in different applications. We found that using a gene-specific primer for RT (the anti-sense QPCR primer) demonstrated the most sensitive QPCR amplification, in terms of lower Ct/Cp values. However, gene-specific priming limits the variety of applications a cDNA pool can be used for. Interestingly, cDNA synthesis occurred without the presence of any primer, although the Ct/Cp value obtained with QPCR was considerably higher than QRT-PCR in the presence of primers. We demonstrated that a blend of anchored oligo-dT with random hexamers, provided a versatile platform in a wide range of QRT-PCR

applications. We have also developed a new proprietary enhancer for the ABsolute QRT-PCR mixes. It can be used during the QRT-PCR reaction, eliminating the need for initial time-consuming DNase treatment as well as avoiding harsh DNase inactivation conditions. The enhancer increases the consistency and improves the overall QRT-PCR efficiency of each reaction by overcoming the inhibitory effects caused by contamination with DNA.
In conclusion, we have demonstrated that improving the efficiency of reverse transcription, with the correct choice of primers and enhancers, can significantly improve the sensitivity of subsequent QPCR.

Session

New Applications: Single Cells

Time: 6th Sep 2005, 08:00:00 - 6th Sep 2005, 10:10:00
Session Chair: Prof Mikael Kubista
Location: lecture hall 14 (HS 14)

Gene expression profiling in single cells.

Anders Ståhlberg[1], Martin Bengtsson(1, 2), Patrik Rorsman(2, 3) and Mikael Kubista[1] (anders.stalberg@tataa.com)
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The transcriptional machinery is ultimately controlled in individual cells by a relatively small number of molecules, which may result in stochastic behaviour in gene activity. Due to technical limitations in the current collection and recording methods, most gene expression measurements are carried out on populations of cells and reflect average mRNA levels. The cellular distributions of transcript levels remain undisclosed although they determine the biological activities in the cells. We have measured gene expression levels of some target genes in individual cells from the pancreatic islets of Langerhans in mouse, and human embryonic stem cells by combining the patch-clamp recording technique and real-time PCR. We find that the transcript levels of the different genes are log-normally distributed. Hence, the geometric mean of expression levels reflects better the gene activity of the average cell than the arithmetic mean measured on a cell population. Ins1 and Ins2 expression was highly correlated in the individual pancreatic beta-cells, while it was not correlated to the expression of the other target genes. The potentials and pitfalls of quantitative single cell mRNA analysis by real-time PCR will be shown.

Quantitative single-cell RT-PCR and calcium imaging in acute brain slices.

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The investigation of quantitative changes in cell type-specific gene expression patterns at the single-cell level can help to understand the molecular basis of physiological roles of specific cell types. Here, we have established a quantitative reverse transcriptase-PCR approach for the analysis of RNA transcript levels in individual cells of living brain slices. Quantification is achieved by using rapid-cycle, real-time PCR protocols and high-resolution external cDNA standard curves for the gene of interest. The method consists of several procedures, including cell soma harvest, reverse transcription and an optimized cDNA purification step, which allowed us to quantify transcripts in small types of neurons, like cerebellar granule cells. Thus, we found that single granule cells contain on average 20 transcript copies of the 'housekeeping' gene glyceraldehyde-3-phosphate-dehydrogenase. We combined two-photon calcium imaging and quantitative RT-PCR in single Purkinje and granule cells, respectively, and identified distinct glutamate receptor-dependent calcium responses in these two cell types. The approach was further tested by profiling the expression of the ionotropic glutamate receptor subunits NR2B and NR2C in the cerebellum. Our study revealed a developmental switch from an average of 15 copies NR2B/cell at postnatal day 8 (P8) to about 5 copies of NR2C/cell after P26. Taken together, our results demonstrate that the new method is rapid, highly sensitive and provides reliable results in neurons of various sizes. Our method can be used in combination with calcium imaging, opening the possibility of systematic analyses of functional parameters in a network of cells in relation to cell-specific mRNA levels. At present, this method is one of the most sensitive and efficient approaches for combined functional-molecular analysis in multicellular networks.

Amplification Based Assays in Nanoliter Volume Range.

Andreas Dahl[1], Marc Sultan[1], Regine Schwartz[1], Matthias Lange[1], Alexander Jung[2], Michael Steinwand[2], Kenneth Livak[2], Hans Lehrach[1] and Lajos Nyarsik[1] (dahl@molgen.mpg.de)
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2: Applied Biosystems.

Even after 20 years of its invention, the polymerase chain reaction (PCR) plays a central role in many applications in molecular biology. By using fluorescent dyes to monitor the accumulation of products, the so-called real-time or quantitative PCR (qPCR) makes the quantification of initial target molecules possible. This opens a wide range of possible applications.
For its use in high-throughput-screening we developed a platform for strongly miniaturized performing of qPCR. We show results of qPCR in 200nl reaction volumes. Our systems shows a high sensitivity down to the single molecule level. Reliable quantification could be shown down to 10initial target molecules per 200nl reaction. For the feasibility of high-throughput screening studies a workflow has been developed, which considers a minimal risk of cross contamination and a high thoughput.
We will present results from a tissue specific expression profiling study, performed on the newly developed platform in 200nl final reaction volume.
Therefore a set of 19 genes was choosen. Five different tissues from mouse were screened for differentially expressed genes. Evaluation of data was done by comparison with data from standard assays in 10µl volume.

Forensic and Single-Molecule Assays of Mitochondrial DNA Using LATE-PCR.

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LATE-PCR invented in our laboratory is an advanced form of asymmetric PCR for efficient amplification of substantial concentrations of single-stranded molecules that can be probed and sequenced by a convenient "Dilute-'N-Go" procedure. Using these techniques we have now developed assays for the both hypervariable sequences, HV1 and HV2, within the control region of human mitochondrial DNA (mtDNA). The resulting amplicons, 549 and 464 base pairs, are longer than those generated in typical symmetric PCR assays, in accord with the underlying properties of LATE-PCR. We are using these assays to demonstrate accurate, inexpensive, and rapid amplification and sequencing of very small forensic samples of human hair and even single finger prints. When combined with additional technologies developed in our laboratory these assays are sensitive and specific down to single mtDNA genomes, and thus can be used to study mtDNA heteroplasmy, as well as changes in heteroplasmy as a function of disease, aging, diet, and environmental toxicity.

Session

New Applications: New methods

Time: 6th Sep 2005, 10:40:00 - 6th Sep 2005, 12:30:00
Session Chair: Ph.D. Russell Gene Higuchi
Location: lecture hall 14 (HS 14)

Rapid Development of RT-PCR Assays for RNAi Experiments Using Pre-designed LNA-probe Libraries.

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Genomic approaches that promise to characterize gene function on a genome-wide scale require reliable and rapid methods to quantify gene expression levels. A key advance in function genomics has been the use of RNA interference (RNAi) which allows the silencing of genes in metazoans through introduction of short double-stranded RNAs homologous to endogenous target mRNAs. RNAi has become a widely used approach to deplete endogenous genes and to study the phenotypic effects of knocked down components of many pathways implicated in physiology and disease. Stringent quality control procedures to monitor RNAi experiments are essential to measure knock-down efficiency and assess the strength of phenotypic changes. Requirements for such a methodology are (i) high flexibility in assay design if many different genes need to be examined, (ii) precision in assessing gene expression levels and (iii) ability to measure expression levels of multiple genes in parallel. A significant technical challenge remains a versatile design of RT-PCR assays and the necessary experimental optimization of assays. To perform RT-PCR experiments on large scale it would be desirable to combine the flexibility of a SYBR green assay with the specificity of hybridization probes. We will discuss experiments how versatile assays that measure the mRNA concentration of target genes can be developed using the Drosophila Universal ProbeLibrary and its application for RNAi experiments.

A Multiplex Branched DNA Assay for Parallel Quantitative Gene Expression Profiling.

Michael Flagella*, Son Bui*, Zhi Zheng, Cung Tuong Nguyen, Aiguo Zhang, Larry Pastor, Yunqing Ma, Wen Yang, Kim Crawford, Gary K. McMaster, Frank Witney and Yuling Luo (yluo@genospectra.com)
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We describe a novel method to quantitatively measure mRNA expression of multiple genes directly from crude cell lysates and tissue homogenates without the need for RNA purification or target amplification. The multiplex bDNA assay adopts the branched DNA (bDNA) technology into the Luminex fluorescent bead-based platform through the use of cooperative hybridization, which ensures exceptionally high degree of assay specificity. Using in vitro transcribed RNA as standards, we demonstrated that the assay is highly specific, with cross-reactivity less than 0.1%. Our results also showed detection sensitivity of 25,000 RNA transcripts and with intra-plate and inter-plate coefficients of variance (CV) of less than 10% and 15%, respectively. Using three ten-gene panels for proinflammatory and apoptosis response, we demonstrated highly specific multiplex expression profiling directly from cell lysates. The assay data highly correlates with measurements obtained using the single-plex bDNA assay with a correlation factor of 0.94. The multiplex bDNA assay thus provides a powerful means to quantify the gene expression profile of a defined set of target genes in large sample populations.

TRIPLEHYB: A Novel Detection Format for real-time PCR.

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In this study a novel detection format for use with real time PCR called TRIPLEHYB probe format is presented. The TRIPLEHYB format besides a conventional set of primers comprises a pair of labeled oligonucleotide probes. Both probes consist of a target-complementary subsequence and a target-unrelated subsequence which has the capability to form a stem structure between the probes. In the presence of target the upstream probe hybridizes by its target-complementary 5` end and the downstream probe adjacently with its 3` target-complementary end to the desired sequence. Simultaneously, an intermolecular stem structure is formed between the 3` end of the upstream and the 5` end of the downstream probe thus stabilizing the structure of the detection complex. In such a manner stabilized complexes may be either cleaved by the 5` -> 3` exonuclease activity of Taq DNA polymerase after primer elongation thus separating e.g. reporter from the quencher dyes introduced into one or both probes or may be used together with the hybridization probe formate technology in that a donor and an acceptor dye are brought into close proximity.
Examplary for detection of Hepatitis B Virus (HBV) in reference plasma we compared the TRIPLEHYB assay with a related TaqMan™ assay. Preliminary results demonstrate that the TRIPLEHYB assay works as reliable as the TaqMan™ assay. There is also some evidence that the novel detection formate seems to be less susceptible to inhibition thus resulting in higher efficiency (about 30%) of HBV quantification as revealed by analyzing a human plasma sample with known viral load.
In conclusion, the novel detection format presented in this study could be useful for a wide range of real time PCR applications including multiplex real time PCR.

Correlation of microarray and quantitative real-time PCR results.

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Microarray techniques allow the parallel assessment of the relative expression of thousands of transcripts in response to different experimental conditions or in different tissues. The ability to correctly identify differentially expressed genes is limited due to the signal to noise ratio, variation in the levels of gene expression, and/or the variability in the measurements due to the assay itself. Therefore, unequivocal identification of differentially expressed transcripts must depend on an independent confirmation. Quantitative real-time RT-PCR (QRT-PCR) is the method of choice because of its broad range of linearity. Furthermore, it can be easily adapted to study systematically tens to hundreds of different transcripts.
I will talk about different microarray platforms and how they differ in their characteristics during the process of hybridization. I will discuss the general differences between microarray hybridizations and PCR reactions. In particular, I will compare the performance of different platforms such as a custom cDNA microarray, a commercial oligonucleotide array and QRT-PCR. Genes present on both arrays, including nearly equal numbers of regulated and unregulated transcripts were assayed for their relative expression. The resulting fold changes were used to compare both platforms to each other. The rank order of differentially expressed genes were similar but the correlation of their fold changes were only modest. A comparison with the QRT-PCR data showed a strong tendency to underestimate the fold changes of the differentially expressed genes for both platforms.
Studies of the transcriptome in tissues are more demanding than cell culture experiments due to the heterogeneity of the tissue. Most tissues contain different cell populations that are closely intermingled. The expression of any specific gene may be restricted to a subpopulation of cells and changes in gene expression may thus occur in only a small fraction of the cells expressing that transcript. These dilution effects can result in relatively low levels of expression for many genes in tissue homogenates. Biologically significant differences in expression may result in only small fold changes. I will talk about the effects of increasing tissue complexity on detection of differentially expressed transcripts in focused microarray studies using a mouse cell line, mouse hypothalamus and mouse cortex. As tissue complexity increases, distinguishing significantly regulated genes from background variation becomes more difficult. However, when an adequate number of replicate experiments were performed low-level expressed transcripts could be identified using microarrays and confirmed by QRT-PCR.

Session

New Applications: Multiplexing

Time: 6th Sep 2005, 13:30:00 - 6th Sep 2005, 16:00:00
Session Chair: Professor Stephen Andrew Bustin
Location: lecture hall 14 (HS 14)

The best of both worlds - New dyes for qPCR for use in combination with probes.

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Currently in real-time PCR one commonly chooses between using a sequence specific probe or an unspecific double stranded DNA binding dye for the detection of the amplified DNA. The sequence specific probe has the advantage that it only detects the correct product, while the unspecific dye has the advantage that a melting curve can be performed after the run, revealing what kind of products have formed.
Here we present the new strategy to use both a sequence specific probe and a new unspecific dye, BOXTO, in the same reaction, taking advantage of the best sides of both chemistries. We show that BOXTO can be used together with both TaqMan probes and LNA probes, without interfering with the PCR or the probe chemistries. The melting curve analysis derived with the BOXTO dye clearly shows the products' melting behavior and can be used to identify primer-dimer formation and anomalous samples.

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qPCR pitfalls - primer and probe design / fluorophore quencher combinations.

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Eurogentec, Belgium.

When designing a qPCR assay, different parameters have to be taken into account such as samples/experimental group, type of controls, design and synthesis of the primer and probe, method of RNA extraction (quality and purity of RNA), reverse transcription (one step or two step) and qPCR (singleplex or multiplex). All these steps are commonly discussed except the design of probes and primers.
Primer and probe design is a crucial step in your experimental design; time spent in the design of the primers and probes will save time in the optimisation of the assay. Design, not only includes the basic rules, pitfalls or tricks to design primers and probes but also a combination of the right fluorophores and quenchers: which fluorophore should be chosen, which quencher should be combined, how to multiplex them. These choices are not just dependant on your assay and your instrument but also on the available synthesis techniques. Design pitfalls and tricks for various instruments and fluorophore-quencher combinations will be discussed.

Going MULTI – how to easily achieve high multiplexing in real-time PCR.

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Research and Development, QIAGEN GmbH, Deutschland.

This presentation will focus on how to overcome the challenges of quantitative, multiplex, real-time PCR and RT-PCR. Issues that will be discussed include:
• The use of optimal combinations of cations and additives in the PCR buffer to provide specific and enhanced annealing of primers to nucleic acid templates
• The use of a hot-start DNA polymerase and inhibition-relieving agents to improve multiplex, real-time, one-step RT-PCR asays which were previously poor in sensitivity and specificity
Data showing successful multiplex analysis of up to 5 targets will be presented, and optimal combinations of reporter dyes for multiplex analysis will be described.

Plexor™ Real-Time Quantitative PCR Systems: Multiplexed assays made easy.

Kyle Hooper (kyle.hooper@promega.com)
Promega Corporation, United States.

Join Promega to learn more about our new Plexor™ Real-Time qPCR and qRT-PCR Systems. The Plexor Systems work by measuring a reduction in a fluorescent signal during amplification. Amplification uses only two primers, one containing a fluorescent tag and a modified base. As amplification proceeds, fluorescence is reduced by site-specific incorporation of a fluorescent quencher inserted opposite a complementary modified base in one of the primers. The quencher is in close proximity to a fluorescent dye located on the end of the primer, resulting in a reduction in the fluorescent signal. After PCR, a melt analysis can be run to provide an internal control for the final assay design or to expedite troubleshooting during development. Plexor Reactions require only two primers for each target. Multiplex assay design is further simplified by the use of web accessible Plexor Primer design program specifically engineered for multiplex assay design. See how the Plexor Technology can be applied to multiplexed gene expression analysis and SNP genotyping. Understand how the system will simplify your assay development, give you the ability to run one chemistry on any real-time PCR instrument and enhance your data analysis with the straightforward data analysis software.

Two-color multiplex assay for the identification of Orthopoxes viruses with Real-Time LUX PCR.

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2: Invitrogen, United States.

The LUX system (Light Upon eXtension) is a real-time detection platform that can be used for detecting and assaying pathogen nucleic acids. This system is PCR- based that uses one self-quenched fluorogenic primer labeled with a single fluorophore. The labeled primer emits fluorescence upon its incorporation and extension into the target nucleic acid sequence, and the fluorescence intensity is proportional to the amount of nucleic acids amplified during the PCR reaction. In this study, a highly sensitive and specific assay for identifying orthopox viruses was developed. The genomes of Orthopoxvirus species are extremely conserved and require a technology that can offer flexibility to enable high level of specificity. We used a variation of the LUXTM detection system, named Universal LUX platform. This technology enables the design of primer sets in the best area for detection specificity without following the design rules that apply to regular LUX primers. The assay is a real-time multiplex Universal LUX-PCR targeting the hemagglutinin gene sequence designed to allow simultaneous detection of Variola and other orthopox viruses. The detection limit of the assay was 50 and 100 copies for plasmid and genomic DNA, respectively, which represents 0.1 to 10 fg of DNA per reaction. These detection limits were highly reproducible. Regression analysis showed that the assay had linearity over seven logs with 0.97 correlation coefficient. The sensitivity and specificity were determined using a panel that consisted of 100 samples and controls. Both sensitivity and specificity were rated at 98%. Thus, the assay offers a sensitive, specific and quantitative tool for simultaneous detection of Variola and other orthopox viruses.

Session

New Applications: Mixed session

Time: 6th Sep 2005, 16:30:00 - 6th Sep 2005, 18:30:00
Session Chair: Msc. Tzachi Bar
Location: lecture hall 14 (HS 14)

ChIP studies on a HOX gene regulated by Polycomb group and trithorax group proteins.

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Polycomb group (PcG) and trithorax group (trxG) proteins act as antagonistic epigenetic regulators to maintain transcriptional OFF and ON states of target genes. PcG proteins exist in multimeric protein complexes and act through specific cis-regulatory silencer sequences, called Polycomb response elements (PREs), that are critically required for PcG silencing. The mechanism of this repression is only poorly understood but three lines of evidence suggest that PcG repressor complexes do not simply compact target gene chromatin and thereby render them inaccessible to general transcription factors. First, PRC1, one of the known PcG protein complexes is associated with numerous dTAFIIs. Second, X-ChIP studies in tissue culture cells revealed that general transcriptional factors are associated with promoters repressed by Polycomb group proteins. Third, In transgenic assays it was shown that PcG silencing does not interfere with the recruitment of RNA polymerase II to promoters.
To better understand the mechanism of PcG repression, we performed an extensive X-ChIP analysis combined with qRT-PCR detection in which we compared the binding of PcG and trxG proteins to Ubx, one of the HOX genes, in its OFF and ON state in imaginal disc cells of Drosophila larvae.
We made several unexpected observations. We find that most PcG and trxG proteins are bound to Ubx both in the OFF and in the ON state. Strikingly, PcG and trxG proteins are also bound to a downstream PRE that is located within the transcribed region of the gene. However, even though PcG and trxG protein are bound in both states, we find that the histone methylation patterns are fundamentally distinct between the ON and the OFF state, suggesting regulated enzymatic activities of the responsible HMTases. In particular, in the upstream control region, the repressive methylation marks H3K27me3 and H3K9me3 are present in both the OFF and the ON state, whereas in the transcribed portion of the gene, these modifications are only present in the OFF state and are absent in the ON state. By contrast, the ON state but not the OFF state is characterized by methylation of histone H3 at lysine 4 in the beginning of the transcribed region of the gene. Finally, we find that RNA polymerase II and general transcription and elongation factors are bound to the Ubx promoter both in the OFF and ON state, but we find that elongation factors are present on the transcribed gene only in the ON state. This suggests that PcG repressors block transcription by interfering with an early step in transcriptional elongation.

The Ups and Downs of Gene Regulation: Validating siRNA Gene Expression Disruption with RT-qPCR.

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RNA interference (RNAi) or post-transcriptional gene silencing (PTGS) is a phenomenon where dsRNA specifically blocks the expression of its homologous gene. The advent of siRNA-directed 'knockdown' has sparked a revolution in somatic cell genetics, allowing for the inexpensive and rapid analysis of gene function in mammals. Coupled with data from genome projects in various organisms, siRNA-directed gene silencing has the potential to allow for the determination of the function of each gene that is expressed in a cell-type- or pathway-specific manner. There are many ways to deliver the siRNA including electroporation, Biolistics™, microinjection and lipid-mediated methods. Due to its low cost, simple protocol and consistent results, we used siLentFect™ for lipid-mediated delivery of siRNA to study the mammalian polyamine biosynthesis pathway, an important pathway for cellular proliferation. Ornithine decarboxylase (ODC) was chosen as a target due to its key role in the polyamine biosynthesis pathway. ODC has been identified as a potential therapeutic target in diseases such as cancer, where control of cellular growth can benefit afflicted patients. Polyamines stabilize membrane and cytoskeletal structures and are involved in the synthesis of DNA, RNA and protein. RT-qPCR data will be presented demonstrating the knockdown of ODC expression and the concomitant effects on other genes in the pathway.

Real-time immuno-PCR for quantification of proteins.

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As real-time PCR attracts more and more users, new applications are being developed. One of the most recent and powerful ones is real-time immuno-PCR for sensitive quantification of proteins. An immobilized antibody is used to capture the specific protein, which is then bound by a second antibody that is labelled with a DNA molecule. After careful washing to remove any non-specifically bound components the amount of DNA-label is quantified with real-time PCR. This corresponds to the amount of the specific protein that was present in the sample. Compared to ELISA, which is the common protein quantification method, real-time immuno-PCR is more sensitive and has much larger quantification range.
Real-time immuno-PCR assays can be assembled in different ways. We have compared three assemblages that differ in how the capture antibody is bound to the tube surface and how the DNA-label is attached to the detection antibody. Assemblage I is assembled stepwise attaching the capture antibody to the vessel surface by adsorption and the DNA-label is linked to the detection antibody through biotin and streptavidin. In assemblage II capture antibody is also adsorbed to the vessel surface but the detection antibody is pre-conjugated to the DNA-label. Finally assemblage III also uses the pre-conjugated detection antibody/DNA-label, but here the capture antibody is bound to surface via biotin to immobilized streptavidin. We found assemblage II to be the most sensitive, while assemblage III was the most reproducible

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microRNA expression profiles from Real-time PCR classify ES and differentiated cells.

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TaqMan® miRNA assays have been developed using stem-loop primers for reverse transcription (RT) followed by real-time PCR. A total of 252 mouse and/or human miRNA assays were tested with four different mouse embryonic stem (ES) cell lines and their differentiated embryoid bodies (EBs) and six mouse tissues. Of 238 human miRNAs examined, 215 (90%) were detected in mouse tissues. MicroRNA expression profiles or fingerprints can classify the ES cells, differentiated EBs and adult tissues. MicroRNA expression levels globally increase upon ES cell differentiation. We have identified a number of ES-specific and differentiation-related miRNA candidates that could be used as molecular markers to determine ES cell identity and to monitor its spontaneous differentiation. There exists significant variability in miRNA expression among four ES lines, suggesting that some ES cell cultures may contain a variable portion of spontaneously differentiated cells. Pre-amplification based TaqMan miRNA assays are being developed to detect all miRNAs from a single ES cell. Single-cell miRNA profiles will be invaluable in exploring stem cells and the different types of cells that stem cells can differentiate into.

Session

Normalization

Time: 6th Sep 2005, 08:00:00 - 6th Sep 2005, 10:10:00
Session Chair: Neven Zoric
Location: lecture hall 15 (HS 15)

Normalization of gene expression: state of the art and preview on a new strategy using expressed Alu repeats.

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With the advent of accurate gene expression technologies such as real-time quantitative RT-PCR, the requirements for proper reference genes to normalize the data (this is elimination of experimental, non-specific variation) have become increasingly stringent. Although many studies have reported that reference gene expression can vary considerably among samples and conditions, only few studies have addressed the critical issue of true reference gene validation. A comparative overview will be presented on the various methods that have been described to assess reference gene expression stability. We will explain in more detail our geNorm framework for the ranking of candidate reference genes according to their stability, for determination of the optimal number of reference genes in a given experimental setup (depending on the sample heterogeneity), and for normalization using the geometric mean of at least 3 validated reference genes.
While above cited methods work well in identifying proper reference genes, they require relatively extensive experimental work to do so. To address this issue, we and others are currently exploring alternatives for reference genes. With the knowledge that some human genes contain one or more repeat sequences, we performed an_in silico_analysis of the human genome sequence. This indicated that a few thousand Alu repeat elements belonging to more than 30 families are expressed in as many as a few thousand different genes (predominantly in their 3' untranslated region). By designing primer pairs that selectively amplify different Alu repeat families, we are thus simultaneously measuring the expression of many different transcripts, acting as an estimation of the general mRNA fraction abundance. Data will be presented on how expressed Alu repeat elements can be applied in accurate, experimental-validation-free normalization of primate gene expression.

Normalisation of mRNA levels against total DNA content.

Shu-Rui Li [1], Doug Storts [2], Becky Hands [1], Benjamin Krenke [2], Ethan Strauss [2], William Ogunkolade [1], Stephen Andrew Bustin [1] (s.a.bustin@qmul.ac.uk)
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2: Promega Corporation, USA.

Normalisation of qRT-PCR data against three or more internal reference genes is generally accepted as the most appropriate, and universally applicable method for ensuring appropriate quantitative comparison between different samples. However, comparing mRNA levels from different tissues or individuals, or investigating the effects of a variety of extracellular stimuli on gene expression requires extensive validation and revalidation, and may result in the need to screen large panels of reference genes. DNA content, on the other hand, is largely invariant, and is an obvious candidate for use as an internal reference. However, there are serious issues concerning inhibition of the qRT-PCR step by DNA, and most RNA extraction protocols aim to minimise the amount of DNA contamination carried over into the RNA sample. Nevertheless, for some applications normalisation against DNA targets may be useful. We have used Promega's new Plexor chemistry to develop a normalisation strategy based on a fourplex assay. It targets four DNA-specific markers that are not amplified or deleted in colorectal cancer. This method is shown to be useful as an alternative normalisation strategy for the quantification of mRNA levels in normal colon and colorectal cancer biopsies.

Normalization genes for heart failure myocardium in mice, rats and humans.

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3: Center for Heart Failure Research, Faculty of Medicine, University of Oslo.

Real-time quantitative reverse-transcriptase PCR (QPCR) is a sensitive method that gives the opportunity to quantify mRNA in very small tissue samples. At the same time, the sensitivity of the analysis imposes strict requirements on how to compare expression data between samples. The most common strategy has been to normalize the expression of a specific gene to a housekeeping gene across all samples, assuming that the expression of the housekeeping gene is invariant. The variation in the expression of the reference gene would therefore reflect variation in the sample preparation, sample input and other experimental variables. In particular, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), beta-actin and 18S ribosomal RNA have been the most frequently used reference genes.
We have evaluated the variability of 8 candidate normalization genes (Arbp0, Rpl32, Rpl4, Tbp, Polr2a, Hprt1, Pgk1 Ppia) and compared them to GAPDH and 18S ribosomal RNA using Taqman assays in mouse and rat post-infarction heart failure models as well as in human explanted failing hearts. Both normalization genes and methods were evaluated by calculating the relative changes in commonly measured genes cited in the heart failure literature such as atrial natriuretic factor, Serca2 calcium ATPase, the sodium calcium exchanger (Ncx1) and phospholamban (PLB). The expression level of each gene was normalized to each of the 8 candidate normalization genes as well as GAPDH and 18S ribosomal RNA. Expression variability was evaluated by both Genorm (Vandesompele et al, Genome Biol. 2002, 3:research0034.1-0034.11) and REST (Pfaffl et al, Nucleic Acids Res. 2002;30(9):e36.
In general, Rpl32, Rpl4, Polr2a and Tbp are good candidate genes for normalization in cardiac tissue, depending on the expression level of the test gene. Gapdh also varies little in mouse and rats myocardial samples. However, in humans, there is a larger variability in Gapdh expression, thus precluding the use of Gapdh as a normalization gene in myocardial tissue from explanted human hearts.

Early mouse development and mammalian embryonic stem cells: a qRT-PCR story.

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The understanding of the signaling pathways during mouse embryogenesis and its application on directing embryonic stem (ES) cells towards specific cell types has become an important topic in the past few years. In addition, the method of relative mRNA quantitation by qRT-PCR has also been introduced to developmental biology. Some of the major applications of qPCR in this field will be presented:
First, we have analyzed 10 potential reference genes in the developing mouse embryo and in differentiating mouse and human ES cells with both geNorm and Normfinder (Willems et al Submitted to Stem Cells). We propose a single stable reference gene for each of the individual systems analyzed. However, we could not identify common stable reference genes for the three systems together, for which we suggest to use a normalization factor based on multiple reference genes.
Second, we have studied the expression of all Wnt genes and their potential antagonists in the developing mouse by qRT-PCR. New expression patterns were found in blastocysts and postimplantation embryos and were confirmed with in situ hybridization (Kemp et al Dev Dyn. 2005 Jul;233(3):1064-75).
Third, the application of qRT-PCR on directed ES cell differentiation will be discussed. In the lab, mesoderm (future muscles, blood,…) and ectoderm (brain, central nervous system,…) induction are studied on mouse ES cells. Several growth factors including Wnts and their antagonists have crucial roles in this differentiation towards mesoderm or ectoderm. The specific fate of the differentiated cells is then studied by the expression of genes correlated with that specific fate.

In conclusion, the use of qRT-PCR in developmental biology will be illustrated with several research topics in the lab and advantages and limitations of its applications to developmental biology and stem cell research will be discussed.

Session

Optimization - part 1

Time: 6th Sep 2005, 10:40:00 - 6th Sep 2005, 12:50:00
Session Chair: Bob Rutledge
Location: lecture hall 15 (HS 15)

Design and optimization of Taqman and SYBR Green I real-time qPCR assays.

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The price of real-time qPCR instrumentation has reached a level where more and more individual laboratories can afford to purchase a machine. This has greatly expanded the number of individual users of real-time qPCR. However, the number of investigators experienced in real-time qPCR is not keeping pace. One of the earliest challenges for new users is the design of a new assay for their research project and the proper utilization of that assay. Although there are many qPCR assays commercially available, these readymade assays are not necessarily the best solution for every project and for those working on non-mammalian systems, not applicable. Another related topic in assay design is whether to use an external standard for quantification or present data as a fold difference. The role of standards in assay quality control and their subsequent use in assays will be discussed. This presentation will be focused on 3 areas: 1) explaining the basic design rules for probe-based and non-probe based assays; 2) presenting examples of proper assay quality control using examples of different design challenges; and 3) comparing different standard types for the same assay.

Comparison of MMP gene expression analysis by capillary and "realplex" real-time PCR.

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Objectives: To compare capillary and "realplex" real-time PCR for the analysis of matrix metalloproteinase 2 (MMP-2) and MMP-9 gene expression in non-stimulated synovial fibroblasts (SFB) and macrophages (mf) from rheumatoid arthritis (RA) or osteoarthritis (OA) patients, as well as in TNF-alpha-stimulated, monocyte-like THP1 cells.
Methods: Total RNA was isolated (kit system; Macherey & Nagel) and cDNA was synthesized (Superscript II; Invitrogen) from purified primary-culture RA- and OA-SFB (n = 6 each) or from RA- and OA-mf (n = 3 each). Using the "LightCycler Fast Start DNA Master Sybr Green I" kit (Roche; reaction volume 20 mikrol) and dilution series of cloned plasmid DNA as external standards, MMP-2/9 expression was determined using identical conditions for capillary and "realplex" real-time PCR (protocol 1). In addition, MMP-9 expression in non-stimulated and TNF-alpha-stimulated THP1 cells (n = 1) was analyzed using a self-designed PCR kit (Hot Master Taq, Eppendorf; Sybr Green, Molecular Probes; bovine serum albumin, New England BioLabs) and identical conditions for capillary and "realplex" real-time PCR (protocol 2). In both experimental series, MMP expression was normalized to the expression of the house-keeping gene GAPDH.
Results: Highly reliable and comparable standard curves were obtained for GAPDH (slope approx. -4.0; r2 > 0.990), MMP-2 (slope approx. -5.0; r2 > 0.995), and MMP-9 (slope approx. -3.6; r2 > 0.996) using protocol 1. Almost identical results were obtained for all genes in RA- and OA-SFB and mf using capillary and "realplex" real-time PCR. Highly reliable and comparable standard curves were also obtained for GAPDH (slope approx. -3.5; r2 > 0.998) and MMP-9 (slope approx. -3.7; r2 > 0.997) using protocol 2. Except for one case, almost identical results were observed for GAPDH and MMP-9 using capillary and "realplex" real-time PCR in non-stimulated and TNF-alpha-stimulated THP1 cells.
Conclusion: Comparative analysis of MMP-2/9 expression in RA- and OA-SFB (mf) or non-stimulated/TNF-alpha-stimulated THP1 cells by capillary and "realplex" real-time PCR yields highly reliable and comparable standard curves, as well as almost identical results. Larger sample numbers and easier handling procedures may favor an augmented application of "realplex" real-time PCR in the future.
This study was supported by the German Federal Ministry of Education and Research (BMBF; grant FKZ 01ZZ0105 and 01ZZ0405 to R.W. Kinne, Interdisciplinary Center for Clinical Research Jena) and the German Research Foundation (DFG; grant KI 439/7-1 to R.W. Kinne), as well as a grant for the advancement of female scientists to Elke Kunisch (LUBOM Thuringia).

Finding the needle in the haystack - LNA bases enhance SNP detection dramatically.

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Sensitive qPCR detection of low copy numbers is sometimes still a challenge, but achievable. The background nucleic acid in a sample may interfere compared with 'synthetic' plasmid-based situations, but as long as the background is basically non-related, qPCR should catch one to ten copies. Much different is the situation where search for a variant of mutation in the background of 'wildtype' sample.
We applied competitor oligonucleotides containing Locked-Nucleic-Acid (LNA) bases in our established 'Clamped-Probe-Assay' using 5-nuclease (TaqMan) or LightCycler hybridization probes for the detection of highly diluted mutations. Depending on the individual application we were able to detect to the point of 10 copies of a single base variant in a background of 1,000,000 wildtype genome equivalents, running a simple two-step PCR protocol, taking far less than one hour.
We will present actual data for the detection of substitution and deletion mutations, including examples from cancer- and leukaemia diagnosis, mutation detection in DNA isolated from urine, detection of minimal amounts of resistant Mycobacteria from mixed cultures and prenatal allele-typing of fetal DNA isolated from maternal blood.

Infectious disease diagnostic research in Africa; the role of real time PCR.

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Infectious diseases are a major cause of mortality and morbidity in the developing world. The Boxing Day tsunami that hit Asia at the end of last year is reported to have killed ~300,000 individuals; HIV/AIDS, Tuberculosis and Malaria have this same effect every three weeks. In a paediatric post-mortem study performed in Lusaka, Zambia 2002 investigating respiratory illness none of the investigated children had a correct diagnosis prior to their death. The required therapy was available it was the diagnosis that let them down. Diseases like tuberculosis are notoriously difficult to diagnose, research into this area is of paramount importance especially with the recent, well publicised global initiatives to improve access to therapies like anti-retrovirals for HIV; without accurate methods of diagnosing and monitoring these diseases these initiatives will be seriously compromised.
The ability to improve diagnostic procedures for infectious diseases has recently been identified as the most important role for biotechnology in the developing world. We are using real time PCR at two levels for diagnostic and prognostic assessment of tuberculosis and other respiratory infections. The first level uses qPCR to measure host and pathogen factors associated with infection (e.g. immune responses, genetics etc.) and the second level investigates the presence of pathogen nucleic acid as a marker for infection of disease.
While real time PCR may never be truly applicable as a simple diagnostic test performable in the field, the techniques sensitivity, reproducibility and versatility provide an important role in this field of research and diagnostics. With our collaborative network we have also incorporated this technology as part of our technology transfer program with four of our African partners. This greatly reduces the

problems associated with sample storage as there are none of the issues associated with sample transport to western laboratories, but more importantly means that it is the local African scientists who are performing much of the work.
Conventional and real time PCR diagnostic procedures for diseases like tuberculosis have not been very successful and are generally not used routinely as a diagnostic method, certainly not in the developing world. There are many reasons for this but poor assay design and laboratory procedures are often key factors. This is confounded by the fact that PCR is seen as a single entity (i.e. poor efficacy of a particular assay is considered poor efficacy of PCR) and the stages required to get a result (sampling, extraction and PCR) are rarely studied individually. We are approaching this problem by using in silico methods to assist the assay design and internal controls to provide confidence in our results. Our diagnostic and prognostic studies using real time PCR can provide important clinical tools that will be important in the management of diseases like tuberculosis.

Session

Optimization - part 2

Time: 6th Sep 2005, 13:50:00 - 6th Sep 2005, 15:20:00
Session Chair: Prof. Dr. Heinrich H.D. Meyer
Location: lecture hall 15 (HS 15)

Relative real time PCR for gene expression measurement in breast cancer biopsies.

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Introduction: Currently real time quantitative PCR (qPCR) is the most precise tool to measure gene expression. Whilst it is widely used in breast cancer research, a number of specific issues should be addressed before the technique can be employed into clinical evaluation of breast cancer specimens, including the following:
 Tissue processing:
Stabilization in RNA-later complicates tissue cutting for histology. To reconcile histological evaluation and RNA preservation the biopsies can be collected and stored in liquid nitrogen, frozen tissue immersed then in OCT and section taken for histology. After this the whole block of tissue in OCT can be processed in RNA-later-ICE, tissue taken out of OCT and RNA extracted with standard methods.
 Reference genes:
10 candidate reference genes stably expressed in breast cancers were identified using literature search and own microarray data (215 breast biopsies before and after hormonal treatment [1]). Candidate genes were validated in qPCR on a separate set of 38 biopsies. The most stable expression was observed for KIAA0674, TBP and PUM1. Geometric average of these reference genes can be recommended for measurements of hormone-dependant gene expression in breast cancer specimens.
 Data processing:
An optimised operator-independent standard data processing procedure should be employed (e.g. [2]). Variation in intra-assay PCR replicas can be used for intra-assay quality assessment.
 Repeatability assessment:
In academic research the uncertainty of qPCR can be overcome by accumulating appropriate sample size. This can not be applied for individual qPCR measurements for potential clinical purposes. In this case the repeatability coefficients can be applied. To estimate the sources of uncertainty and individual repeatability of qPCR measurements, paired biopsies were taken from 9 tumours; two reverse transcriptions (RT) were run then on each of RNA and two PCRs on each cDNA. Expression of 2 different genes was measured (CCNB1 and SCGB2A2). Observed coefficients of repeatability were 2.2 and 15 times respectively. Exclusion of a small number of obvious outliers dramatically improved the coefficients of repeatability (to 1.3 and 3.1 times respectively). The components of variance analysis showed that 1% of the total variance was introduced by PCR, 4% – by RT and 10% – by sampling. 85% of observed variation reflected true differences between tumours.
Conclusion:
Reliability of qPCR results is very dependent on pre- and post- PCR steps. The whole procedure of gene expression measurement – from tissue acquisition to statistical interpretation – should be thoroughly standardised and adopted to the specifics of clinical breast specimens in order to employ the potential of qPCR for molecular tumour profiling in breast clinic.
References:
1) Miller et al J Clin Oncol 2005 v23, N 16S, Pt I, 198s, 3025
2) Larionov et al BMC Bioinformatics 2005 Mar 21;6(1):62

The Fitness of an Amateur Football Team: Using High Resolution Melts to Determine Genotypes.

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In the endeavour for Citius (faster), Altius (higher), Fortius (stronger), the athlete has turned to science to aid them in achieving their best. With the advent of human genetics we are beginning to understand that sports performance can be influenced by the make up of our DNA. Genetic polymorphisms have been associated with sporting phenotypes such as the ACTN3 gene, which has been linked to muscle fibre development, a key element in determining if an athlete is more suited to power or endurance (Yang et al. 2003). The detection of single nucleotide polymorphisms (SNPs) through Real-Time PCR has involved the use of complex probe systems such as dual labelled hydrolysis probes (TaqMan) or fluorescence resonance energy transfer (FRET) hybridisation probes. Both systems require complex designs and optimisation strategies. However, with the introduction of more sensitive and non-inhibitory intercalating dyes such as LC Green (Wittwer et al. 2003), SNP detection has been made significantly more user friendly and cost effective. The aim of the study was to evaluate the fitness characteristics of several amateur footballers (n = 12) and compare their physiological profiles (VO2 max, lactate levels, body fat composition and power levels) to their genetic profile of four sports associated genes (hypoxia inducible factor 1, monocarboxylate transporter 1, beta-2 adrenergic receptor and alpha actinin 3). The determination of each genotype was achieved using High Resolution Melt (HRM) analysis on DNA obtained from buccal swabs. HRM involved using primers designed to amplify smaller (60 - 80bp) than conventional (150 - 200bp) product sizes, and LC Green intercalating dye (Idaho Technologies) in a modified Rotor-Gene thermal cycler (Corbett Research). Following amplification the products were melted at 0.1°C increments over a melt domain no greater than 10°C. Normalisation of the melt curves allowed for genotype differentiation, with the difference between Tm values ranging from 0.2°C – 0.8°C. Heterozygotes produced two distinct slopes with the curves shifting to lower temperatures due to formation of heteroduplexs. Detection of wild type from mutant was determined by a shift in the melt curve to either lower or higher temperatures depending on the base pair substitution. Players that were mutant for the ACTN3 gene (n = 2) showed greater power output compared with other genotypes (n = 9). Similarly, MCT1 gene mutants (n = 2) showed higher lactate accumulation during peak periods of exercise compared with other genotypes (n = 9). Although, the cohort of players was small, genotype analysis demonstrated some associations with sporting phenotypes including speed and fatigue. The use of genotyping in sport can offer a benefit to athletes by aiding them in improving their physiology based on their genetic make up, accelerating sport performance.

Fast, Sensitive and Reproducible qPCR with Mastercycler ep RealPlex & epMotion Robotics.

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Real-time PCR is now accepted as a precise and reliable method for quantification of nucleic acids. Now, the focus has shifted from the decision of whether or not to use it, to the details of getting an assay up and running: amplification efficiency, speed, accuracy, reproducibility, cost-efficiency. We have tried to address these new needs by creating a real-time PCR instrument that is sensitive as well as fast, a liquid handling robot that is fast, easy-to-use and highly

accurate even at very small volumes, and reagents that minimise optimisation steps. With this combination, our hope is that the user will not be held back by long run times, complicated optimisation setups, and time-consuming pipetting. With issues such as pipetting errors, contamination or user errors a thing of the past, the user is free to concentrate on the salient scientific data of each experiment. Utilising SYBR Green and Black Hole Quencher dual-labelled probes, we will show that it is possible to achieve fast run times and efficient reactions, as well as multiplexing.

Session

Standardization

Time: 6th Sep 2005, 15:50:00 - 6th Sep 2005, 18:30:00
Session Chair: Professor Gregory L. Shipley
Location: lecture hall 15 (HS 15)

A Comparison of Real-Time RT-PCR Technique, Chemistries and Instrumentation in Laboratories Utilizing the Same Assay.

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Standardization of a variety of technologies to improve experimental results has been a goal of the Association of Biomolecular Resource Facilities since its inception. The Nucleic Acids Research Group (NARG) has concentrated its efforts in the area of real-time PCR for the past three years. The goal is to provide educational material and benchmark tests that enable participants to gain positive feedback on their technique, reagents and instrumentation in an anonymous fashion. The study for 2004-2005 entailed making separate 6-log dilution series of an *in vitro* transcribed RNA or a synthetic DNA template template and generating a standard curve for each using the optimized primers (and probe) provided. Each laboratory had the choice of running the assays using either Taqman® or SYBR® Green chemistry and the reagents and real-time instrumentation commonly used in their laboratory. The quality of the data was measured by comparing the slopes, Ct values, r2 and y-intercepts obtained from the two standard curves generated from the DNA versus RNA template. The same templates were used to compare the results obtained from variable settings of the threshold and to evaluate the results obtained by default parameter settings versus operator-adjusted settings on 9 different real-time PCR platforms.

Accurate Gene Expression Analysis with High Flexibility: Concepts and Developments.

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Roche, Deutschland.

Gene Expression Analysis is a field of growing interest. The complete process of generating gene expression data consists of many steps starting from sample to analysis. The data from the gene expression analyses is influenced by biological variations governed by the laws of nature which cannot always be controlled. Nevertheless, a reduction of technical variation is the achievable goal, reached by using workflows that show lowest inter- and intra-assay variances.
Gene Expression Data is generated using systems such as real-time PCR Instruments. In real-time PCR, the PCR reaction is monitored via indirect detection methods, using fluorescent dyes or probe-based formats, which help monitor the amplification process. As a consequence, this necessitates optimization of real-time PCR-Instruments for homogeneity and accuracy in both processes: amplification and detection. The accuracy of amplification is often contrary to speed and homogeneity. Flexibility in the usage of assay format or fluorescent dye requires highly specific optical units.
The Roche LightCycler® 2.0 System Family sets the standard for rapid, sensitive and accurate real-time PCR. With LightCycler® 480 System, we will introduce an additional instrument in the market─ adding to the already well-accepted performance of LightCycler® 2.0 System on a multiwell-plate based platform. In addition to delivering speed and accuracy, the system is highly versatile. The flexibility of the optical system enables using most current dyes, also in multiplex. Exchangeable 96- or 384-multiwell plate block facilitates different throughput adaptations.
The highly flexible software supports multitude-assay formats and applications for quantification as well as melting-curve analysis. The algorithms used to characterize genes qualitatively and quantitatively have been optimized extensively, thereby facilitating the analysis of Gene Expression Data.

The Data Comparability Challenge - Standards and Best Practices.

Morten Tolstrup Andersen, Carole Foy (morten.andersen@lgc.co.uk)
LGC Limited, United Kingdom.

As the technology providing benchmark results in the field of gene expression measurements, quantitative RT-PCR must live up to high expectations. Pre-developed assays and all-inclusive kits make it easy to set up and run a QRT-PCR experiment, and in the standardisation context this may well be useful. Confidence in the data, however, can only be achieved if appropriate assay validation, experimental design and quality assessment have been applied. We are currently evaluating and developing experimental procedures with the purpose of establishing a best practice approach to QRT-PCR based on recent discussions among users in the scientific community.

Putting the "quantity " into quantitative PCR: A simplified approach to the establishment and application of quantitative scale.

Bob Rutledge and Don Stewart (Bob.Rutledge@NRCan.gc.ca)
Natural Resources Canada, Canada.

Founded upon utilizing fluorescence to monitor amplicon accumulation, the fundamental quantitative unit of real-time PCR is fluorescence. Historically, conversion of fluorescence units to the number of target molecules (No) has been accomplished by constructing standard curves for each target, based upon correlating reaction fluorescence (fluorescence threshold, Ft) to cycle number (threshold cycle, Ct). What has been generally overlooked, is that alternative methods for establishing quantitative scale can be developed, in which reaction fluorescence is directly correlated to DNA mass. This approach can be illustrated by considering the classic exponential equation for Ct-based qPCR:
No=Nt/(E+1)^Ct
where Nt is the number of amplicon molecules at threshold and E is the amplification efficiency. The slope of a standard curve provides an estimate of amplification efficiency, whereas Nt is determined from the intercept, allowing Ct to be converted to No (Rutledge and Côté 2003). Importantly, application of this equation is not restricted to using molecule number as the quantitative unit; DNA mass can also be used:
Mo=Mt/(E+1)^Ct
where Mo is target quantity expressed as DNA mass, and Mt is the mass of amplicon DNA at fluorescence threshold. This concept can be extended to fluorescence units:
Fo=Ft/(E+1)^Ct
where Fo is target quantity expressed in fluorescence units. An important advantage of this last equation is that target quantity determination only requires an estimate of amplification efficiency, in that Ft and Ct are known entities. Thus, if reaction fluorescence can be converted to DNA mass (via a method termed optical calibration), the number of target molecules can then be calculated based upon amplicon size (Rutledge 2004). This presentation will describe the development and evaluation of the quantitative accuracy, of various approaches that can be used to correlate reaction fluorescence to DNA mass, as modeled upon SYBR Green I fluorescence and lambda genomic DNA. Among the many advantages of this approach, abrogating the preparation of a quantified standard for each target has some of the greatest practical implications.

R. G. Rutledge, C. Côté, Nucleic Acids Res. 31, e93 (2003).
R. G. Rutledge, Nucleic Acids Res, 32, e178 (2004).

Assay standardisation using universal internal controls and lyophilized reagent beads.

Andreas Eckelt (aeckelt@cepheideurope.fr)
Cepheid SA, Deutschland.

For better comparability of results, standardisation of Real Time PCR assays is very important. Cepheid lyophilised reagent beads together with sample preparation controls and the newly developed universal internal control provide the basis for more reliable assays. Based on this bead technology Cepheid has developed a versatile ASR reagent line to be followed soon by FDA cleared and CE marked clinical tests for applications in microbiology, virology and tumour diagnostics.

Session

BioInformatics

Time: 7th Sep 2005, 08:00:00 - 7th Sep 2005, 12:10:00
Session Chair: Dr. Michael W. Pfaffl
Location: lecture hall 14 (HS 14)

From Sequences to Synthesis: Optimal Amplification through Careful Oligonucleotide Selection.

Ben Sowers (ben@biosearchtech.com)
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Successful real-time PCR experiments are based on a series of careful decisions. This includes the design of high-performing primers against the sequence of interest, the matching of fluorophores and quenchers to the optics of the instrument, and finally the choice of purification stringency to apply to the fluorescent-labeled oligo. For each step of this process, we will discuss factors that influence assay performance and explore newly available internet tools that offer guidance. This includes a tutorial of an advanced, web-based, software program to design *TaqMan*® and *Amplifluor*® assays against a submitted sequence. Applying sophisticated algorithms towards primer and probe selection, this software offers scrutiny against primer-dimers, secondary structures, and non-specific amplification. Focusing the software on a panel of target genes from the human genome confirms it's ease of use and subsequent performance in real-time PCR.

Estimation of sample specific efficiency - methods and applications.

Tzachi Bar[1], Ales Tichopad[2], Mikael Kubista[3], Michael W. Pfaffl[4] (tzachi.bar@gmail.com)
1: Chalmers University of Technology, Department of Chemistry and Bioscience, Göteborg, Sweden.
2: LabonNet, Tivon, Israel.
3: TATAA Biocenter, Göteborg, Sweden.
4: Physiology Weihenstephan, Center of Life Science, Technical University of Munich, Germany.

For proper quantification of nucleic acids by real-time PCR, compared samples should have similar PCR efficiencies. However, the abundance of PCR inhibitors suggests that this might not be the situation in all cases. Last few years brought a list of publications dealing with this issue, some of them describe new methods for estimation of sample specific efficiency, and some describe procedures based on sample specific efficiencies, as absolute and relative quantification, replacement of standard curve efficiency, and kinetics Quality Assurance (QA) tests. However, the usability of these procedures depends on the closeness of the estimated efficiency to exponential phase efficiency and precision of efficiency estimation. In this work we used modular SAS-based software to investigate 5 published methods for estimation of sample specific efficiency and compare their precision and closeness to exponential phase efficiency. Analyzing over 750 samples from 3 platforms we conclude that (i) quantification with sample specific efficiencies might be too inaccurate and imprecise for most uses, and often will require local adjustment of efficiency estimation; (ii) replacement of standard curve efficiency and kinetics QA may be useful.

Amplification efficiency dynamics and its implications: Developing a kinetic-based approach for quantitative analysis.

Bob Rutledge (Bob.Rutledge@NRCan.gc.ca)
Natural Resources Canada, Canada.

Application of sigmoidal mathematics has lead to new insights into the kinetics of real-time PCR amplification. Paramount is recognition that amplification efficiency is dynamic such that each cycle has a unique amplification efficiency, referred to as “cycle efficiency” or Ec (Rutledge 2004). In this presentation, a new sigmoid-based model for PCR amplification is introduced, that was derived from kinetic analysis in which Ec is correlated to reaction fluorescence. In addition to allowing PCR amplification to be modeled with unprecedented precision, new insights into the kinetics of PCR amplification were also achieved. These include the ability to mathematically predict entry into the plateau phase, which was found to be determined by a combination of amplicon accumulation and initial amplification efficiency. Variation in reaction volume, initial amplification efficiency and amplicon size provide direct experimental support for this new model, which further illustrates that the progress reduction in Ec is likely associated with self-annealing of amplicon strands. Of practical significance, this new approach to kinetic analysis allows both initial amplification efficiency and target quantity to be determined directly from the fluorescence readings generated by an individual amplification reaction. Combined with its amiability to automation, the computational simplicity of this new sigmoidal model could provide an effective, easy to implement alternative to the exponential-based threshold methods that currently predominate.
R. G. Rutledge, Nucleic Acids Res. 32, e178 (2004).

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qBase: relative quantification software for management and automated analysis.

Jan Hellemans, Geert Mortier, Anne De Paepe, Frank Speleman, Jo Vandesompele (Jan.Hellemans@UGent.be)
Center for Medical Genetics, University Hospital Ghent, Belgium.

Gene expression analysis is becoming increasingly important in biological research and clinical decision making, with real-time quantitative PCR becoming the method of choice for expression profiling of selected genes. Advancements in chemistry and hardware have made the practical performance of real-time quantitative PCR measurements feasible for most laboratories. However, accurate and straightforward mathematical and statistical analysis of the raw data as well as the management of large and growing data sets have become the major hurdles in this type of PCR based gene expression analysis. Since the software provided with the different detection systems does not provide an adequate solution for these issues, we developed qBase, a free software program for the management and automated analysis of real-time quantitative PCR data. qBase is a collection of macros for Microsoft Excel and uses a proven delta-Ct relative quantification model with PCR efficiency correction and multiple reference gene normalization. The qBase Browser allows data storage and annotation by hierarchically organizing real-time PCR runs into projects, experiments, and runs. It is compatible with the export files from many currently available PCR instrument softwares and provides easy access to all your data, both raw and processed. The qBase Analyzer contains an easy run (plate) editor, performs quality control and inter-plate calibration, converts Ct values into normalized and rescaled relative quantities with proper error propagation, and displays results both tabulated and in graphic format. The program can handle an unlimited number of samples, genes and replicates, and allows data from multiple runs to be processed together (preceded by an inter-run calibration if required). The possibility to use up to 5 reference genes allows reliable and robust normalization of gene expression levels. qBase allows easy exchange of data between users, and exports data for further statistical analyses using other dedicated software.

Early Phase Fluorescence Fitting of real-ime PCR reaction.

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Laboratoire de Pharmacologiqe Chimique et Génétique, France.

A mathematical model for PCR early phase modelization has been built using Michaelis-Menten law. The equation derived from this model to fit real-time PCR fluorescence curves gives immediate access to the quantity of initial template target sequence with great precision over a wide scale of template dilution. It has also been shown to be robust to efficiency discrepancies. Such an approach thus offers an advantageous alternative to Ct-based quantifications for quantitative PCR. As this new method provides a mean to ease and secure quantitative PCR experiments with both more reliable results and a lighter procedure, its automation could open the gate for real-time PCR spread outside of research laboratories, towards clinical or industrial fields.

Classification of real-time PCR data.

Mikael Kubista(1, 2), Björn Sjögreen[2] & Amin Forootan[2] (mikael.kubista@tataa.com)
1: TATAA Biocenter, Gothenburg, Sweden.
2: MultiD Analyses, Gothenburg, Sweden.

By real-time PCR gene expression can be measured with much higher accuracy than with alternative techniques such as microarrays. Further, one typically only measures expression of genes that are regulated and, hence, contribute with significant information about the samples. Such experimental data are most suitable for classification. In my talk I will describe the most common classification methods including principal component analysis, hierarchical clustering, self organizing networks, neural networks and partial least-square analysis. I will also demonstrate classification of both samples and genes based on real-time PCR expression data using the software GenEx available on: www.multid.se.

The real-time PCR primer and probe database RTPrimerDB: a major update.

Filip Pattyn, Piet Robbrecht, Jelle Verspurten, Anne De Paepe, Frank Speleman, Jo Vandesompele (Filip.Pattyn@UGent.be)
Center for Medical Genetics Ghent (CMGG), Ghent University Hospital, Ghent, Belgium.

The power of real-time PCR for the quantification of nucleic acids mainly lies in the use of well designed and experimentally validated primers and probe sequences. To prevent time-consuming primer design and evaluation, and to introduce a certain level of uniformity and standardisation among different laboratories, we have developed a public accessible web-based database (RTPrimerDB) to enable the storage and retrieval of oligonucleotide sequences (Pattyn et al., Nucleic Acids Research, 2003). Since its start-up, RTPrimerDB has grown continuously and now contains 3500 real-time PCR assays submitted by 500 registered users.
The primer and probe information can be easily accessed by a simple query on organism and gene symbol or RTPrimerDB ID. In addition, detailed searches can be performed based on gene name or symbol, detection chemistry, application, primer or probe sequence, PubMed ID or submitters name. A search results page links to the assay reports with gene information, assay details, publication information, potential users' feedback and submitter details.
In the latest release of the database a *gene expression assay viewer* is introduced. This feature displays the mapping of primer and probe sequences onto the different transcript variants, along with known SNP positions and peptide domain information. The occurrence of SNPs in the region where the primer/probe anneals and the exact location of an amplicon with respect to the gene exon structure or the protein domains can be examined at a glance, along with an assembly of other available assays for the same gene.
Also new is a module to _automatically evaluate the secondary DNA structure_ of a PCR amplicon for all available assays, as this has been shown to be a critical factor for the efficiency of a PCR reaction. This work is based on Zuker's mfold server (Nucleic Acids Research, 2003).
This new quality control feature is extended with the introduction of an *experimental assay evaluation* questionnaire for primer sequence submitters and assay users. Submitters are obliged to provide details about experimental quality control parameters obtained before the implementation of a new assay. In addition, users who tested an assay from the database can give their valuable feedback on assay performance. Both the experimental evaluation details provided by the submitter as well as user's feedback will allow a better assessment of the reliability of an individual assay.
The last new feature is the introduction of an _in silico assay evaluation analysis pipeline_ for user designed (but not yet submitted) assays. This module streamlines quality assessment prediction prior to experimental evaluation, based on established in silico tools, such as BLAST specificity search, mfold secondary structure analysis, presence of SNPs or plain sequence errors, and graphical visualization of the aligned primer sequences on the target gene.
RTPrimerDB is available at http://medgen.ugent.be/rtprimerdb/

Session

GMO Analytics & Food Hygiene

Time: 7th Sep 2005, 08:00:00 - 7th Sep 2005, 12:10:00
Session Chair: PhD Christiane Albrecht
Location: lecture hall 15 (HS 15)

Keynote Lecture:

Uncertainities and certainties in GMO analytics using qPCR.

Philipp Hübner (philipp.huebner@kl.bs.ch)
Kantonales Labor Basel-Stadt, Switzerland.

The determination of the accuracy of an analytical method represents an important step in the method validation process. The accuracy consists of two components called trueness and precision. Whereas the determination of the precision of a given method such as a qPCR based method is experimentally accessible, the determination of the trueness is often, not only with qPCR based methods, difficult by conception. By definition the trueness reflects the deviation from the true analyte concentration in the investigated sample. However, often the true value of an analyte concentration in a given matrix cannot be determined. This is especially the case with the analysis of the GMO content of foodstuff since the determination of the trueness depends on the availability of certified reference material. Due to the fact that such material is nowadays only commercially available for a few GMO commodities the determination of the trueness still represents a major obstacle in GMO food analysis.
The determination of the accuracy of a method used for the enforcement of the food legislation is necessary at the legally defined limits such as labelling threshold limit or other limit value. For GMO food analysis the effective labelling threshold limits are around 1% of GMO per ingredient. There are not many regulation where a limit value is restricted to the food ingredient and is not valid for the whole food sample. This legal speciality of the GMO labelling of food leads to a variable limit of quantitation (LOQ) depending on the investigated food sample. Whereas in absolute terms the limit of quantitation (LOQ) can be estimated to be around 50 copies of the target DNA segment, the relative limit of quantitation depends in a linear fashion on the amount of the corresponding ingredient in the investigated food sample. More precisely, the LOQ of qPCR based GMO detection methods depends on the amount of the DNA from a given plant species present in the analytical sample used for qPCR. For determining the GMO concentration in a food ingredient the ratio of the number of gene fragments derived from a GMO and of the number of gene fragments common to a given plant species such as soybean is determined. Since this process includes two qPCR based determinations the overall uncertainty of the measurement increases. In addition, the measurement of GMO concentration is restricted to foods containing only one soya and/or one corn and/or one canola (etc.) ingredient.
Using a theoretical approach we tried to estimate the contribution of different factors such as pipetting, PCR efficiency and fluorescence measurement to the overall uncertainty of qPCR based detection methods. Clearly, pipetting is not the main source of uncertainty!

Accurate GMO quantification in food samples.

Dörte Wulff (d.wulff@genescan.com)
Eurofins Genescan, Deutschland, www.genescan.com.

The safety, authenticity and appropriate labelling of food products are of pivotal importance for consumers, manufacturers and traders in the food chain. As in the case of GMOs (genetically modified organisms) PCR based methods have become the assays of choice when it comes to analysis and control. Also, for the purpose of quantification, real-time PCR assays have been developed to detect GM-plant derived ingredients e.g. from soy, corn, beet, cotton, tomato, papaya etc. The presentation will give an overview which factors have to be considered to ensure reliable results as a PCR based assay comprises several stages from sampling, test portion preparation, DNA extraction and PCR. Also the overall assay format including its controls and the criteria for the final interpretation of result play a crucial role. All these stages and elements interact and can introduce error or otherwise affect the results and quality of the analysis. In addition to this, the method validation is critical and if not carried out properly - does potentially not reveal a lack of performance of a given method. In test portion preparation the main challenge is representative sampling and homogeneity. Efficient DNA extraction and purification methods are important prerequisites for real-time PCR assays. The multitude of different matrices represented in the value chain including products on the market is challenging as the different matrices often do contain different levels and classes of PCR interfering and inhibiting substances which need to be removed efficiently. Further, quantification assays have to provide for high specificity, high reproducibility and high sensitivity. Labelling threshold testing is required from the EU and intended to assure reliable and comparable data, validation and standardization of assays on an international scale (e.g. CODEX, ISO, EU). A test should provide accurate DNA copy numbers in absolute or relative quantities. Relevant performance and validation criteria will be presented including experiences from projects related to current EU-legislation.

The USDA/GIPSA Proficiency Program: A Summary of Participants Capabilities for Detecting and Quantifying Transgenic Events in Corn and Soybeans.

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USDA/GIPSA, United States.

Consumers globally, especially in Europe, demand the right to choose between transgenic, also known as genetically modified organisms (GMO) and non-GMO derived products. Identifying transgenic varieties of foods, by using food labels, is considered the most appropriate means for providing consumer choice in those countries that support this policy. Recently, the European Union revised its food regulations to require labeling as genetically modified if more than 0.9 % w/w, of a food product or raw ingredient is genetically modified. One of the major challenges facing scientists that test for the presence of transgenic events in grains and oilseeds in foodstuffs is how to standardize testing procedures. To ensure comparable analytical results by different laboratories, analysis should be carried out with validated methods using standard reference materials such that precision, accuracy, sensitivity, specificity and robustness can be optimized. In order to establish such procedures, the USDA/GIPSA currently offers a proficiency program which enables participants to monitor their own distinctive method for detecting GMO's. Samples of ground corn or soy material, either fortified to a precise concentration with a particular transgenic event or non-fortified material, are provided on a bi-annual basis to participating laboratories. No methodologies are specified, and organizations can use either DNA- and/or protein-based testing technologies. In October 2004, sixty organizations participated in the program.

- Twenty-six participants submitted qualitative only results
- Three participants submitted quantitative only results
- Thirty-one participants submitted a combination of qualitative and quantitative results.

A summary of participant's results relative to event fortification levels is discussed. The events with the consistently most accurate quantifications (i.e., % relative errors ≤ 20%) at all fortification levels were: CBH 351, BT-11, TC1507 and NK603. Yet, this was only observed on four of the nine transgenic events. There seems to be some degree of difficulty with achieving accurate quantification values when PCR is deployed to detect the presence of transgenic events in grains and oilseeds. GIPSA will continue to play an active role, in providing standardization for the grain industry, as a synergistic approach toward application and detection methods progress in biotechnology-derived food products.

Application of synthetic DNA-Standards for the quantitative screening of different genetically modified rapeseed lines via real-time PCR.

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Bayerisches Landesamt für Gesundheit und Lebensmittelsicherheit, Deutschland.

Real-time PCR based assays for the quantitative screening of different genetically modified rapeseed lines in foods and feeds were developed and validated. The systems were designed to allow the detection of different construct-specific junction regions in herbicide tolerant rapeseed lines as well as the detection of the taxon-specific acetyl-CoA carboxylase *BnACCg8*- gene. Quantitative analyses were based on the application dilution series of synthetic DNA standards. These were obtained according to a novel technique, which allows the generation of hybrid DNA molecules carrying respectively one copy of the GMO-specific and the taxon-specific target sequences. This analytical strategy allows a relative quantification of the copy numbers of the recombinant DNA in relation the copy numbers of a taxon-specific gene in terms of haploid genomes. The performance and the suitability of these quantitative screening systems for purposes of routine testing were demonstrated in an in-house validation study. Analyses were carried out using mixtures of genomic DNA from conventional and genetically modified rapeseeds with defined GMO-contents (1.8, 0.9, 0.09 and 0.045%).
The project is funded by the Bavarian State Ministry for the Environment, Public Health and Consumer Protection.

Cloned plasmid DNA molecules as a tool for GMO analysis.

Isabel Taverniers, Marc De Loose (i.taverniers@clo.fgov.be)
Department of Plant Genetics and Breeding, DvP-CLO, Melle, Belgium.

European Commission Recommendation 2004/787 on technical guidance for sampling and detection of GMOs, accompanying the new Regulations (EC) 1829/2003 and 1830/2003, clearly defines a GMO expression unit as "the number of target DNA sequences per target taxon specific sequences calculated in terms of haploid genomes". This recommended unit definition reflects the acceptance in the EU of the DNA-based PCR technique as a reference method for regulatory compliance. Real-time PCR based estimation of GMO percentages involves a 'double quantification': a GMO specific DNA target is determined relative to a plant species or taxon specific sequence. The unit of expression should be consistent with the unit of measurement, which is linked to the type of calibrants used for quantification. In practice, if a GMO content is to be expressed as a ratio of genome copy numbers, this requires calibration standards to be expressed also in copy numbers.
Based on the theoretical basis of DNA and GMO quantification, I describe the setup and experimental testing of a new concept, i.e. the synthesis of plasmid DNA standards for GMOs. For a number of commercialized transgene plants, an event-specific DNA fragment as well as a plant specific sequence were generated, cloned into a plasmid vector and transformed to competent bacterial cells. The number of target copies, or haploid genome equivalents, was calculated from the measured plasmid DNA concentration. In this way, we obtained a dilution series of plasmid DNA, usable for setting up absolute standard curves in real-time PCR. The concept was experimentally tested by quantifying real GMO samples with well-known relative contents of each event. The relatively new plasmid DNA calibrants can be compared to the traditional, genomic DNA calibrants, isolated from matrix reference materials (RMs). To test the utility of plasmid DNA molecules, extensive comparative and commutability studies were carried out with (1) single target plasmid (STP) calibrants, (2) multiple target plasmid (MTP) standard sets, and (3) genomic DNA RMs. I discuss some applications of plasmid DNA markers for GMO analysis, as well as major advantages compared to other types of standards.

Detection of Food Pathogens using the Smart Cycler II.

Martina Fricker[1], Ingrid Huber[2], Siegfried Scherer[1], Monika Ehling-Schulz [1] (martina.fricker@wzw.tum.de)
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2: Bayerisches Landesamt für Gesundheit und Lebensmittel, LGL, Oberschleißheim, Germany.

Real-time PCR is beeing more and more applied to the detection of foodborne pathogens. This method is also increasingly taken into account with respect to the methods suggested by §35 LMBG. Up to now, several real-time PCR kits have been available from different manufacturers for the detection of *E. coli* , *Salmonella* , *Listeria* , … These kits are often optimized for a certain real-time PCR machine but can also be used with others. Features like the ramp rate or the optical detection system (especially the built-in filter set) can influence the sensitivity / applicability of these kits. In our case, we compared the Bio-Rad iCycler and the Stratagene Cycler with the Smart Cycler II from Cepheid. The latter machine has 16 independently programmable reaction sites with a maximum heating rate of 10°C / sec. This ramp rate is also programmable so that the Cepheid Smart Cycler II can be slowed down to the ramp rates of other real-time cyclers, thereby mimicking their cycling conditions.
At the moment no commercial kits are available for the detection of *Bacillus cereus* This is an emerging foodborne pathogen that is increasingly identified as the causative agent of food poisoning and food spoilage. Two types of gastrointestinal diseases caused by *B. cereus* can be distinguished: emesis and diarrhoea. In general, the symptoms of both types of food poisoning are mild and disappear within 24 hours, but there are also exceptional cases with more severe symptoms and even several deaths have been reported. Based on the genetic information of the emetic toxin (cereulide) synthesis, real-time PCR assays with SybrGreen and with a specific Taqman probe were established. Both systems were optimized to the Smart Cycler II, but also tested under the conditions that occur in the Stratagene and Bio-Rad iCycler.

Poster presentations:

Session

Poster Session: Pre-Analytical Steps

Time: 5th Sep 2005, 18:30:00 - 5th Sep 2005, 20:00:00
Session Chair: Prof. Dr. Heinrich H.D. Meyer
Location: Student Cafeteria

P01

A New Mini-prep and Rapid DNA Extraction Method.

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DNA extraction is an important step in molecular assays and plays a vital role in obtaining high-resolution results in gel-based systems, particularly in the case of cereals with high content of interfering components in the early steps of DNA extraction. Here we report a new rapid mini-prep DNA extraction method, optimized for rice, which was achieved via creating some modifications in present DNA extraction methods, especially in first step of breaking down and lyses of cell wall, and the use of cheap and frequent chemicals, found in every lab, in the next steps. The normal quality and quantity was obtained by the method. The PCR based assays also revealed the efficiency of the method. The advantages of this method are:
1: it is applicable with both dry and fresh samples,
2: no need to large weight samples,
3: no need to liquid nitrogen and
4: easy, rapid and applicable in every laboratory.

P02

The complex study of storage and handling of human placenta tissue samples.

Martina Pejznochova, Marketa Tesarova, Tomas Honzik, Hana Hansikova, Jiri Zeman (martina.pejznochova@centrum.cz)
Charles University, Czech Republic.

Human term placenta is the most obtainable tissue resource without invasive surgery to patient for study of mRNA expression in human fetal tissue.
The aim of the study is to determine appropriate options for quantification of mRNA expression by real-time RT-PCR in human fetal tissue.
We collected the set of twenty tissue samples of human term placentas, seven of them we used for the pilot time-course study of RNA degradation in two different temperatures. Various instruments NanoDrop ND - 1000 (NanoDrop Technologies), Experion (Bio-Rad Laboratories), Agilent 2100 Bioanalyzer (Agilent Technologies) were used for analysis of RNA quality, quantity and purity. Acquired RNA was transcribed into cDNA and specific mRNAs were amplified by classical PCR and then by real-time RT-PCR. According to first results it appears that the critical limits for storage and handling of tissue samples from human placenta are temperature and time period before freezing. Next samples are under investigation and on the basis of all results we will carry out the optimalization of total RNA izolation from human placenta.
Supported by grants GAUK 16/2004c/1.LF1 and GACR 303/03/H065.

P03

RNeasy FFPE Kit - RNA Isolation from Formalin-Fixed Paraffin-Embedded Tissue.

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Formalin fixation has been the method of choice for histopathology applications for decades, and is still widely used. Today, there is an increasing need to recover RNA from such materials, in order to open up the vast archives of formalin-fixed paraffin-embedded (FFPE) tissue samples for modern gene expression methods. Standard methods for RNA isolation from FFPE sections rely on digestion of the tissue by proteinase K, followed by nucleic acid extraction, removal of genomic DNA by DNase digestion, and further cleanup. These methods are time-consuming, expensive, and often poorly reproducible.
A novel procedure is presented that allows the release of RNA, and removal of genomic DNA without enzymatic treatment, and in a considerably shorter time frame than current state of the art. RNA yields from a variety of FFPE tissues are equal to or substantially higher than using other protocols, and the RNA obtained by this new method is a superior substrate in PCR applications. PCR analysis also demonstrates efficient removal of genomic DNA.

P04

Bone marrow samples treated for cytogenetic analysis are not suitable to PCR amplification.

Barbara Rocca, Silvia Calatroni, Elisabetta Lugano, Ilaria Giardini, Jessica Quarna, Marina Boni, Paola Maria Cavigliano, Rita Zappatore, Marilena Caresana, Paolo Bernasconi (s.calatroni@smatteo.pv.it)
IRCCS Policlinico S.Matteo - Division of Hematology, Italy.

Cytogenetics analysis, routinely performed in our laboratory to investigate chromosomal abnormalities associated with hematological malignancies, has produced, in almost two decades of activity, a considerable number of samples, consisting of short term (one day) bone marrow cultured cells stored in methanol/acetic acid. The present study evaluates the possibility to isolate, from these samples, RNA, suitable for PCR amplification, comparing it to ribonucleic acid extracted from conventional PCR sample, consisting of bone marrow mononuclear cells routinely separated by density gradient centrifugation. We considered 10 CLL (Chronic Lymphocytic Leukemia). For all these patients, samples treated for cytogenetic

analysis and for PCR testing were available in correspondence of the same bone marrow withdrawal. All the samples were submitted to total RNA isolation by Qiagen spin column (RNeasy Kit). Five-hundred ng of RNA were reverse-transcribed in cDNA using SuperScript II (Invitrogen) and Random Hexamers (Applied Biosystems). Amplification of 3 different genes (ABL, B2M and hTERT) was performed in duplicate by real-time PCR using SybrGreen Master Mix (Applied Biosystems), specific primers and 1/10 of cDNA volume. In all the patients, DCt (cytogenetic sample – PCR sample) was calculated for each gene. ABL DCt mean value was 5,747 (st. dev.= 2,986), B2M was 3,123 (st.dev.= 0,493) and hTERT -0,826 (st.dev.=1,663). Data analysis with REST software, considering hTERT as target gene and both ABL or B2M as reference gene indicates that in cytogenetic sample hTERT is significantly up-regulated (factor 28,502 with ABL as reference, p value= 0.004; factor 15,444 with B2M as reference, p value= 0,0115), in comparison with conventional PCR sample. This observation in cytogenetic samples may be caused by RNA degradation due to methanol/acetic acid treatment but also by gene expression variation due to short term culture. In conclusion cytogenetic sample are not suitable to be used in PCR amplification, particularly in real-time quantification.

Session

Poster Session: New Application

Time: 5th Sep 2005, 18:30:00 - 5th Sep 2005, 20:00:00
Session Chair: Prof. Dr. Heinrich H.D. Meyer
Location: Student Cafeteria

P05

Expression profiling of early development stages of Xenopus laevis.

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IMG AS CR, Czech Republic.

The development of organisms is a complicated and highly accurate process that eventually leads to mature animals containing hundreds of differentiated cell types. Every cell type originates from a single cell, the fertilized egg. During the development, a cell's fate depends on transcription and translation activities of specific genes. Expression of genes in multicelular organisms is restricted spatially and temporally. Here, we present temporal expression profiles of selected *Xenopus laevis* housekeeping and embryonic genes determined by qRT-PCR. Interestingly, none of the four tested housekeeping genes (EF-1alpha, GAPDH, ODC, L8), which are frequently used as reference genes, is expressed constantly at the same level during *X. laevis* early development. Expression profiles of the 22 tested embryonic genes important for development fall into two classes: (i) Maternally expressed genes, such as VegT, Vg1, and Wnt11, are present from egg to gastrula stages, albeit at varying levels. (ii) The other tested embryonic genes are expressed only after the MBT (mid-blastula transition), which demarks the beginning of zygotic transcription. In general, we found a good correlation between mRNA expression levels and known requirements for translational products of these mRNAs In conclusion, qRT-PCR and *X. laevis* embryos seem to be a highly useful model system for large-scale expression profiling during early development.

P06

Two-color multiplex assay for the identification of Orthopoxes viruses wih Real-time LUX PCR.

Mohamed Aitichou [1], Sandrine Javorschi-Miller [2], Sofi Ibrahim [1], Mark Andersen [2] (sandrine.javorschi@invitrogen.com)
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2: Invitrogen, United States.

The LUX system (Light Upon eXtension) is a real-time detection platform that can be used for detecting and assaying pathogen nucleic acids. This system is PCR- based that uses one self-quenched fluorogenic primer labeled with a single fluorophore. The labeled primer emits fluorescence upon its incorporation and extension into the target nucleic acid sequence, and the fluorescence intensity is proportional to the amount of nucleic acids amplified during the PCR reaction. In this study, a highly sensitive and specific assay for identifying orthopox viruses was developed. The genomes of Orthopoxvirus species are extremely conserved and require a technology that can offer flexibility to enable high level of specificity. We used a variation of the LUX detection system, named Universal LUX platform. This technology enables the design of primer sets in the best area for detection specificity without following the design rules that apply to regular LUX primers. The assay is a real-time multiplex Universal LUX-PCR targeting the hemagglutinin gene sequence designed to allow simultaneous detection of Variola and other orthopox viruses. The detection limit of the assay was 50 and 100 copies for plasmid and genomic DNA, respectively, which represents 0.1 to 10 fg of DNA per reaction. These detection limits were highly reproducible. Regression analysis showed that the assay had linearity over seven logs with 0.97 correlation coefficient. The sensitivity and specificity were determined using a panel that consisted of 100 samples and controls. Both sensitivity and specificity were rated at 98%. Thus, the assay offers a sensitive, specific and quantitative tool for simultaneous detection of Variola and other orthopox viruses.

P07

Global RNAi phenotype analysis for cancer drug target identification and validation by qRT-PCR.

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Quantitative real-time PCR (QRT-PCR) is a widely used technique for accurate evaluation of RNA expression levels. Due to its simplicity, wide dynamic range of quantification, sensitivity, and precision, qRT-PCR is the method of choice for validation of RNAi knockdowns. We describe here a global RNAi phenotype analysis for cancer drug target identification and validation of drug targets by qRT-PCR. Gene regulatory networks that control growth and survival of cancer cells are affected by many intrinsic and extrinsic factors. The molecular and genetic context of a cell defines a particular state of a cell and also determines the relative dependency on certain genes that are essential for growth and survival. To better understand the onco-selective dependencies that arise during the neoplastic process, we have developed and applied global RNAi profiling to discover context dependent vulnerabilities in cancer cells. Specifically, we have used high throughput transfection of 10,000 siRNA, (individually targeting 5000 druggable gene targets) to systematically knock down 5000 genes in parallel, and analyzed the effects on growth and survival in human cancer cells. Multidimensional analysis of various cancer cell line models enables the identification of genes, which were selectively and differentially required for growth. When comparing global RNAi profiles across multiple cell lines with very different genetic background, we were able to identify gene knockdowns that were very selective and cell line specific in their phenotype. To understand the source of selectivity, we began by integrating the RNAi phenotype data with gene expression and gene copy number data from microarray analysis. This revealed that some of these contextual vulnerabilities were associated with DNA amplification, suggesting that they may be etiologically relevant dependencies, but others appeared to be simply dependent on cell type. To gain a deeper understanding on how specific cancer associated perturbations can alter the relative dependency on specific gene and pathways for survival, we used isogenic cell lines that vary only in the expression of a single tumor suppressor gene. Using this model cell line, we were able to identify synthetic lethal RNAi targeting events that selectively killed cancer cells with a specific genetic defect. Finally, we exposed cancer cell lines to various anticancer drugs to model the cellular context of drug response, and screened for RNAi targeting events that appeared to enhance or suppress drug response. Using qRT-PCR and a functional pharmacogenomic screen for genes that affect cancer chemotherapy response, we were able to identify potential functionally relevant genes that could give us a better understanding of the mechanism of drug action. Additionally, this approach can be applied to the identification of functionally relevant candidate markers for predicting drug response,

and candidate drug targets for combination therapy to enhance the response to common cancer drugs.

P08

Universal ProbeLibrary: A new concept for real-time qPCR assays.

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Commercially available pre-validated real-time RT-PCR assays simplify the assay development process, but the time of delivery sometimes causes delays on experimental progress. Furthermore, pre-validated probe based assays lack flexibility, due to the fact that these assays target a specific site in a given transcript. Consequently, quantification of another transcript or splice variant requires a different assay. To simplify and accelerate the cumbersome process of quantitative real-time RT-PCR assay development, the Universal ProbeLibrary concept with pre-validated real-time PCR detection probes, and a new web-based ProbeFinder assay design software, was developed.
The novel and highly flexible concept, based on Universal ProbeLibrary sets of 90 pre-validated real-time PCR detection probes, and a novel web-based assay design software, enables fast and easy design of optimal real-time PCR assays for gene expression analysis. By combining individual Universal ProbeLibrary probes and target-specific PCR primers, the ProbeFinder assay design software is able to design more than 644,000 different assays in the human transcriptome or target 98% of all human transcripts. Beside the human Universal ProbeLibrary set sets for mouse, rat, Arabisopsis, Drosophila ,C. elegans and primates are available so far.
The results presented here demonstrate that the Universal ProbeLibrary detection probes perform equally well in real-time PCR expression assays compared to commercial pre-validated probe-based assays, while simultaneously providing a more flexible platform, since a library of 90 probes can be used to cover the entire human transcriptome. Furthermore, the Universal ProbeLibrary assays showed significantly higher specificity compared to SYBR Green I assays avoiding the possible problems of detecting primer dimers or unspecific amplification. The Universal ProbeLibrary probes combined with the ProbeFinder assay design software provide a robust expression profiling platform for high-throughput expression analysis, especially for validation of microarray data and gene knock-down experiments.

P09

Mapping of homozygous deletions in chromosome 3p regions affected in major epithelial tumors using real-time PCR.

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Localization, identification oncogenes and tumor suppressor genes (TSGs) and their expression studies are one of fundamental approaches to understand the difference between normal and disease tissues in conditions like cancer and differentiating between different types of the disease. qQuantitative real-time PCR is the most effective and precise method for the identification of genomic DNA deletions, duplication, amplification and evaluation of gene expression level.
We searched for chromosome 3p homo- and hemizygous losses in 23 lung cancer cell lines, 53 renal cell and 22 breast carcinoma biopsies using 31 microsatellite markers located in frequently deleted 3p regions. In addition, two STS markers (NLJ-003 and NL3-001) located in the Alu-PCR clone 20 region (AP20) and lung cancer region (LUCA), respectively, were used for real-time PCR. We found frequent (10-18%) homozygous deletions (HDs) in both 3p21.3 regions in the biopsies and cell lines. In addition, we discovered that amplification of 3p is a very common (15-42.5%) event in these cancers and probably in other epithelial malignancies.
Real-time PCR showed that aberrations of either NLJ3-001 or NL3-001 were detected in more then 90% of all studied cases. Homozygous deletions (HDs) were frequently detected simultaneously both in NLJ3 -001 or NL3-001 loci in the same tumor. This fact suggests that TSGs in this regions could have a synergistic effect.
The exceptionally high frequency of chromosome aberrations in NLJ3-001 and NL3-001 loci suggests that multiple TSGs involved in different malignancies are located very near these markers.
Precise mapping of 15 independent HDs in the LUCA allowed us to establish the smallest HD region in 3p21.3C located between D3S1568 *CACNA2D2* gene) and D3S4604 *SEMA3F* gene). This region contains 17 genes.
Mapping of 19 HDs in AP20 region resulted in the localization of the minimal region to interval flanked by D3S1298 and D3S3623 markers. Only 4 potential candidates have been discovered in this interval, namely *APRG1 ITGA9 RBSP3/HYA22* and *VILL* which need to be analysed.

P10

DNA isolation from FTA Mini Cards as a high-throughput method for genotyping of Glutathione S- transferase P1 polymorphisms in workers with asbestosis.

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FTA cards (Whatmann Bioscience) are designed for room temperature collection, shipment, archiving and purification of nucleic acids from a wide variety of biological samples for PCR analysis. Although FTA Mini Cards are very convenient for collecting and archiving blood samples the protocol for DNA isolation recommended by the manufacturer requires repetitive isolation/washing procedures for each single nucleotide polymorphisms (SNP) to be analysed and makes the high-throughput analysis of multiple SNPs on a large number of samples needed for molecular-epidemiological studies difficult.
Our aim was to develop a rapid and high-throughput method for DNA isolation from FTA Blood Cards suitable for genotyping of Glutathione S-transferase P1 (GSTP1) polymorphisms in workers with asbestosis. Asbestos related disorders are among the most extensively studied occupational diseases and although the causal relationship between asbestos exposure and pulmonary diseases was well demonstrated, relatively little is known about the genetic factors involved in the susceptibility of the exposed individuals to development of these conditions.
For isolation of DNA and genotyping capillary blood samples from the finger tips have been collected on FTA Mini Cards (Whatmann Bioscience) from 700 study subjects employed in the asbestos cement manufacturing plant of Salonit Anhovo, Slovenia. Among them 350 subjects were diagnosed with asbestosis while the control group of 350 sex and age matched exposed subjects did not develop asbestosis. The method for DNA extraction from the FTA cards using BloodPrep chemistry on the semi-automated ABI PRISM™ 6100 Nucleic Acid PrepStation (Applied Biosystems) was developed.
Our approach allowed for rapid DNA isolation in a 96-well plate format compatible with a high- throughput SNP analysis. GSTP1 genotyping was carried out using Custom TaqMan SNP Genotyping Assay according to the manufacturer's instructions using a reaction volume of 5µl in 384-well plate with ABI PRISM 7900HT instrument (Applied Biosystems). Other SNPs that may modify the risk for asbestosis can be analyzed by conventional or real-time PCR from the eluted DNA stored at –20oC without the need for the repeated DNA isolation.

P11

qPCR in the elucidation of an insect-endosymbiont relationship.

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Sleeping sickness is a reemerging zoonotic disease that is transmitted by the tsetse fly across sub-Saharan Africa. The tsetse fly is associated with two species of endosymbiotic bacteria, *Wigglesworthia glossinidia* and *Sodalis glossinidius* The latter is thought to play a role in increasing the susceptibility of the fly to infection by trypanosomes, the causative agent of sleeping sickness.
The characteristics of the qPCR technology have enabled the dynamics of this insect-endosymbiont relationship to be revealed for the first time. Here qPCR is used to analyse the growth kinetics of *S. glossinidius* populations throughout the tsetse fly's life cycle. In addition RT-qPCR is used to look at the expression of bacterial genes thought to be involved in the establishment of the symbiosis during the same period.
The successful adaptation of this technique for *in vivo* endosymbiont quantification has enabled further avenues of research into the role of this bacterium in this symbiosis. We test the hypothesis that susceptibility to trypanosome infections correlates to *S. glossinidius* populations in tsetse flies, by using trypanosome-infected flies and qPCR.
These results demonstrate the potential of this technique for studying the interactions within such complex systems.

P12

LightCycler® 480 System: a novel real-time PCR platform for high-throughput qPCR and genotyping.

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Abstract 1:

In recent years, capillary-based LightCycler® Systems have become an established standard for rapid and accurate real-time PCR. Based on innovative technology for heating/cooling and highly accurate analysis algorithms, they enable thermal cycling at high speed and with precisely controlled temperatures, characteristics directly related to the consistency and accuracy of scientific results.
The recently developed multiwell (96/384) plate-based LightCycler® 480 Instrument goes one step further in providing enhanced PCR throughput and flexibility. Key to the system´s speed and accuracy are a completely new designed thermal block and optical system which both minimize inter-well, inter-assay and inter-instrument variability. The LightCycler® 480 System uses a time-tested software algorithm for efficiency-corrected relative quantification and automated, melting-curve based genotyping.
Data on this poster will illustrate how the transfer from a capillary- to a multiwell-based LightCycler® System was technically achieved while maintaining speed and accuracy.
(presentation of LightCycler® 480 System continued on P13 and P14)

P13

LightCycler® 480 System: a novel real-time PCR platform for high-throughput qPCR and genotyping.

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Abstract 2:

In recent years, capillary-based LightCycler® Systems have become an established standard for rapid and accurate real-time PCR. Based on innovative technology for heating/cooling and highly accurate analysis algorithms, they enable thermal cycling at high speed and with precisely controlled temperatures, characteristics directly related to the consistency and accuracy of scientific results.
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Data on this poster will illustrate which components (hardware, software, reagents) contribute to the system´s versatility with regard to possible applications, assay formats and dyes
(presentation of LightCycler® 480 System continued on P14)

P14

LightCycler® 480 System: a novel real-time PCR platform for high-throughput qPCR and genotyping.

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Abstract 3:

In recent years, capillary-based LightCycler® Systems have become an established standard for rapid and accurate real-time PCR. Based on innovative technology for heating/cooling and highly accurate analysis algorithms, they enable thermal cycling at high speed and with precisely controlled temperatures, characteristics directly related to the consistency and accuracy of scientific results.
The recently developed multiwell (96/384) plate-based LightCycler® 480 Instrument goes one step further in providing enhanced throughput and flexibility. Key to the system´s speed and accuracy are a completely new designed thermal block and optical system which both minimize inter-well, inter-assay and inter-instrument variability. The LightCycler® 480 System uses a time-tested software algorithm for efficiency-corrected relative quantification and automated, melting-curve based genotyping.
Data on this poster will illustrate which experimental data are available to demonstrate the system´s performance for quantification and SNP analysis applications in pharmaceutical and academic research.

P15

REAL-TIME PCR GENE EXPRESSION ANALYSIS TO EVALUATE ADDITIONAL MOLECULAR MARKERS IN ACUTE PROMYELOCYTIC LEUKEMIA (APL) WITH MULTIPLE RELAPSES.

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In the present study we performed real-time PCR to analyse mRNA expression variations of three different genes in consecutive samples of 5 APL (Acute Promyelocytic Leukemia) patients, 4 of them with multiple relapses and 1 in clinical and molecular remission. PML-RARa is the molecular marker of APL. FLT3 gene is often involved in internal tandem duplication (ITD) in APL patients; this mutation is associated with a poor disease outcome and increased gene expression. hTERT, the catalytic subunit of telomerase involved in telomere length maintainance, plays a still undefined role in haematological malignancies. The study focuses on the application of real-time PCR to evaluate alternative genes, other than PML-RARa, as additional prognostic molecular markers in APL patients. Gene expression analysis was conducted on 80 total RNA samples, previously isolated from mononuclear cells routinely separated by density gradient centrifugation from bone marrow or peripheral blood. All the samples were also submitted to reverse transcription and qualitative PCR for the detection of PML-RARa rearrangements and FLT3 ITDs mutation. Relative quantifications of PML-RARa (BCR1 and BCR3 isoforms), FLT3 and hTERT transcripts were performed by real-time RT-PCR

using SybrGreen Master Mix (Applied Biosystems). ABL mRNA expression was used to normalize target genes expression. For each assay, amplification efficiency (98-99%) and sensitivity (10-3-10-4) were evaluated by standard curve analysis of 5 serial dilution (1-10-4) of total RNA isolated from samples with known high espression of the gene of interest. Both DDCt method and REST software were applied to calculate target genes mRNA levels, expressed as number fold in relation to a calibrator sample. Specificity of each amplification assay was demonstrated by dissociation curve analysis and by agarose gel-electrophoresis of PCR products. FLT3 and hTERT expression analysis was also conducted on a control group of normal sample. In all the relapsed patients, as expected, PML-RARa levels increase at relapse; PML-RARa and FLT3 show a similar pattern of variations during patients follow-up, but an increased expression of FLT3 does not always correspond to ITD reappearance. hTERT increase seems to precede or to accompany the increments of both PML-RARa and FLT3 expression. In contrast, at diagnosis, the patient in clinical and molecular remission presents high FLT3 levels, decreasing with PML-RARa reduction and not associated with ITD mutations; during follow-up, hTERT and FLT3 remain constantly low. Even if additional patients are undoubtedly required to confirm these observations, real-time PCR is a useful approach to investigate new potential molecular markers.

P16

A potential role for hydrocortisone in the positive regulation of IL-15-activated NK cell proliferation and survival.

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Although glucocorticoids (GC) have been mainly described to act as anti-inflammatory and immunosuppressive drugs, they may also positively influence the immune system. In the present study, we demonstrate for the first time that hydrocortisone (HC), in synergy with interleukin-15 (IL-15), induces a dramatic increase in the expansion of peripheral blood-derived CD56+ cells, favoring the preferential outgrowth of classical natural killer (CD56+CD3- NK) over CD56+CD3+ NKT cells. HC plus IL-15-driven CD56+ cells exhibited an increased potential for cytokine production. Elevated levels of GC-induced leucine zipper protein (GILZ) mRNA were detected in both NK and NKT cells cultured with HC. To estimate GILZ mRNA levels we applied Real-Time PCR using the chemistry of LUX primers. Phosphorylation status of signal transducer and activator of transcription 5 (STAT5) was not affected by the presence of HC in either of the populations. On the contrary, HC differentially affected the IL-2/IL-15R β- and γ-chain surface expression and the phosphorylation levels of extracellular signal-regulated kinases 1/2 (ERK1/2) in IL-15 activated NK and NKT cells. Our data ascribe a novel role to GC on mature NK cell expansion and function and open new perspectives for their use in cellular adoptive cancer immunotherapy.

P17

Advanced Real-Time PCR Assays on the LightCycler480 instrument.

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We show first data for the absolute quantification of mRNA and virus samples as well as multiplex typing assay using 5-nuclease (TaqMan) and LightCycler hybridization probes using the new Roche Diagnostics LightCycler480 instrument.

P18

MicroRNA expression signature in human glioblastoma multiforme brain tumor.

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Expression of 180 human miRNAs was examined using recently developed stemloop primers for reverse transcription (RT) followed by real-time PCR. MicroRNAs can be quantified from as few as single cells or as little as 25 pg total RNA. The CT values correlated to the copy numberover up to seven orders of magnitude. The TaqMan® miRNA assays discriminated between two miRNAs that differed by as little as a single nucleotide, and between mature miRNAs and their precursors. This method allows accurate and sensitive miRNA expression profiling and uncovers precise changes of miRNA expression. Comparing to normal human brain, the glioblastoma multiforme (GBM) tumors have a distinct expression signature of miRNAs. Nearly half of miRNAs showed the reduced expression by > 2-folds. In contrast, only 13% miRNAs had increased expression (>2-folds) in GBM. Expression of miR-10a and miR-10b etc. located within class I HOX and miR-129, miR-139, and miR-153 etc. within class II HOX gene clusters is either elevated or reduced (>10-fold), suggesting that these miRNAs may be involved in brain cancers.

P19

Expression and localisation of fibroblast growth factor (FGF) family members in porcine antral follicles.

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The aim of this study was to investigate the possible participation of fibroblast growth factor family members (FGF1, FGF2, FGF7) and their receptor variants (FGFR1IIIc, FGFR2IIIb, FGFR2IIIc) in porcine follicles during final follicular growth. Classifications of follicles into four groups were created based on follicle diameter (2-3, 4-5, 6-7 and >8 mm) and according to the follicular fluid (FF) estradiol-17b (E) content. The mRNA expression was analysed by block reverse transcription polymerase chain reaction (RT-PCR) and quantitative real-time PCR (Rotor-Gene 3000). The hormone concentration was analysed by EIA, and protein localisation by immunohistochemistry. For immunohistochemistry of FGF2 ovaries were fixed in methanol-pure acetic and embedded in paraffin.
Immunohistochemical staining for FGF2 occurred predominantly in stroma tissue, endothelial cells of blood vessel in theca interna and only weak staining in basal granulosa cells. The mRNA expression data obtained by block RT-PCR were confirmed by quantitative real-time PCR. The mRNA signals for FGF1 and FGF2 increased in large follicle groups with a significant difference only for FGF2. The mRNA expression of FGF7 was high already in small follicle group (2-3 mm), decreased significantly in follicle group 4-5 mm followed by a further significant increase in large follicles (>8 mm). The mRNA signal for the FGFR2IIIb und FGFR2IIIc during finale follicle growth was without any regulatory change. There was a significant down-regulation of FGFR1IIIc mRNA expression in follicle group 2-3 mm, with further up-regulation in large follicle groups. The different expression and localisation of FGF growth factor family members suggest that these local produced factors are involved in process of final growth of the preovulatory follicles. FGFs may be involved in the proliferation of capillaries (angiogenesis) that accompanies the selection and development of the preovulatory follicles, resulting in an increased supply of nutrients and precursors, and therefore supporting growth of the ovulatory follicles in pig ovary.

P20

Expression of mRNA for Apoptotic and Anti-apoptotic Factors during Oestrous Cycle and Pregnancy in Bovine Corpus Luteum.

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The aim of this study was to specify the expression of different apoptotic and anti-apoptotic factor family members in the bovine CL during oestrous cycle and pregnancy. Corpora lutea were accordingly assigned to the following stages; Days 1-2, 3-4, 5-7, 8-12, 13-18, >18 (after regression) of oestrous cycle and of early and late pregnancy (<4 and >4 month). The mRNA expression was detected by conventional RT-PCR and quantitative real time PCR (Rotor Gene 3000). Investigated genes were the ligands of the extrinsic apoptotic pathway and their receptors (FAS-L and FAS antigen), tumor necrosis factor alpha (TNFalpha), its receptor (TNFR1). As representatives of the mitochondrial pathway BAX, BCL-XL and three central caspases (Caspase3, -6, -7) were investigated.
The real time PCR data of FAS-L, TNFalpha as well as TNFR1 showed an increase of mRNA expression from day 1 to 18 of the oestrous cycle followed by a decreasing tendency during pregnancy. The FAS mRNA expression data showed no differences in regulation of CL function during oestrous cycle and pregnancy. Caspases3, -6, -7 were less expressed during pregnancy in contrast to expression during oestrous cycle. The mRNA expression of BCL-XL showed an up-regulation during midluteal phase (days 8-12) and late pregnancy. The mRNA expression of BAX was down regulated during pregnancy. Results of our data suggest an important role of FAS-L, TNFalpha and TNFR1 as the mediators of the extrinsic apoptotic pathway in the bovine CL during oestrous cycle, especially during the CL regression, while the mitochondrial pathway represented through BCL-XL and BAX may play a subordinated role. The Caspases3, -6, -7 are continuously expressed during oestrous cycle in CL, however a down-regulation during pregnancy may be necessary for CL maintenance. In conclusion, results of investigated genes suggest an important role of locally produced apoptotic and antiapoptotic factors for CL function during oestrus cycle and pregnancy in cow.
Supported by the DFG BE 3189/1-3 and Scha 257/14-2

P21

Quantitative expression of bone morphogenetic protein and bone matrix protein in human carotid plaque.

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Objectives. Bone morphogenetic proteins (BMPs) regulate differentiation of progenitor cells towards bone and lipid containing adipocytes. Demonstration of ectopic bone formation and lipid accumulation in atherosclerotic plaques has implicated BMPs and other bone matrix proteins in the pathogenesis of atherosclerosis. We investigated the presence of those mineralization-regulating proteins in atherosclerotic plaques and plasma.
Methods: Fresh-frozen carotid endarterectomy samples were analyzed by quantitative RT-PCR for expression of BMP-2, BMP-4, BMP-6 and matrix proteins osteoprotegerin and osteonectin. Samples of the plaque were also submitted for routine H&E staining and plaque characterization. Chi-square analysis was performed to assess the presence or absence of each transcript relative to a plaque characteristic. ELISA for BMP-4 was performed on atherosclerotic patients and volunteers without a history of atherosclerosis.
Summary of Findings
High concentration of BMP-4 protein ($p < 0.002$) was found in patients undergoing cardiac catheterization for suspected coronary disease (497.9 + 107.8 ng/ml) compared to volunteer controls (35.5 + 28.78 ng/ml). Age correlated strongly r= 0.6 plasma BMP-4 levels. Of 8 carotid plaques tested, three specimens had detectable BMP-6, two specimens had detectable osteoprotogerin, one specimen had detectable BMP-4 mRNA and one specimen had detectable osteopontin. There was no relation between plaque characteristics including detectable calcification and the presence of any of the transcripts. All three BMP-6 positive specimens (100 %) were obtained from male patients.
Clinical Application of Findings
1. Mineralization regulating proteins are present in atherosclerotic plaque and represent a target for slowing the disease.
2. A plasma assay of bone morphogenetic protein may be a useful biomarker for assessing risk of atherosclerosis.

P22

Immunerelevant gene expression of primary bovine epithelial cells in response to different mastitis pathogens.

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Infections of the bovine mammary gland are, in addition to the affliction of the animals, a great economic burden in the dairy industry. A better understanding of the innate immune response of the host could lead to a better selection of mastitis resistant cows or to better prophylactic and therapeutical treatments. The epithelial cells play an important role in the initial immune response to invading pathogens into the mammary gland. For better understanding of this process, epithelial cells were isolated from milk from eight Brown Swiss cows and cultured separately, yet simultaneously. Cells were exposed to heat inactivated gram-positive and gram-negative mastitis relevant bacteria. The immune response was detected by mRNA-expression of important immune modulators like cytokines, acute phase proteins and chemokines, with quantitative real-time PCR (qRT-PCR) using RotorGene. An exposure with 0.5 bacteria per cell (MOI; multiplicity of infection) *Escherichia coli* and *Staphylococcus aureus* led to a significant increase of interleukin-8- (IL8) and tumor necrosis factor alpha (TNF)-mRNA expression in the epithelial cells. On the other hand, in *Streptococcus uberis* treatment 10 MOI were needed for a significant increase of cytokine mRNA. Higher MOI did not increase the mRNA expression of the measured immune modulators in all experiments. A treatment of the cells over different time periods showed a faster significant increase of interleukin-6- (IL-6), IL-8-, granulocyte-macrophage-colony stimulating factor (GM-CSF), and TNF-mRNA expression in *E. coli* treatment. However, the amounts of mRNA were not significantly higher compared to *S. aureus* treatment. Serum amyloid A (SAA) mRNA concentration after a 24h exposure was just with this bacterium significantly increased. In contrast, the immune response to the same amount of *S. uberis* was always lower compared to the other bacteria.
In conclusion, the treatment of cultured cells from different cows with different mastitis pathogens is a useful method to investigate the role of epithelial cells in the early immune response of the mammary gland. With qRT-PCR differences in the immune response could be detected. *E. coli* a bacterium, which causes predominantly an acute course, *S. aureus* predominantly responsible for chronic and subclinical mastitis, and *S. uberis* which can cause both kinds of mammary gland infections, were confronted in this model. All strains induced an increased mRNA expression of the measured cytokines. Interestingly, *S. uberis* always induced weaker reactions, also compared to the subclinical infection inducing *S. aureus* However, *E. coli* seems to induce a faster immune response and this is likely related to the acute cause of infections with this bacterium.

P23

Characterization of a novel suspension cell culture system for bovine oviduct epithelial cells.

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To examine early embryo-maternal communication in vitro, a cell culture system mimicking the oviduct with its peculiar physiological characteristics is a prerequisite. Therefore, a novel short-term BOEC suspension culture was established and validated by ultrastructural characteristics and gene expression analysis using qRT-PCR.
Simmental heifers were slaughtered on day 3.5 after standing heat. BOEC from the ampulla of ipsi- and contralateral oviducts were isolated separately by squeezing along the oviduct with forceps. The purity of epithelial cell culture was tested by immunocytochemistry against cytokeratin filaments and vimentin. Cultures were sampled for light microscopy, transmission electron microscopy, scanning electron microscopy, RT-PCR and Western blotting at 0, 6, 12 and 24 hours after seeding. To study effects of hormone supplementation (10 ng/mL P4 or 10 pg/mL E2), cultures were sampled after 6 and 18 hours of stimulation. Maintenance of the ciliated epithelial cell phenotype and ultrastructural characteristics of cultured cells indicated that the culture system is able to provide cells which closely resemble cells in situ. The mRNA expression of some candidate genes varied immediately after seeding of cells, indicating an adaptation period. However, all investigated genes showed a relatively stable mRNA expression level after 6 hours in culture. qRT-PCR results showed no significant difference in gene expression between cultured BOEC obtained from ipsi- or contralateral oviducts. Differences monitored in vivo are therefore likely to result from countercurrent blood flow between ovary and corresponding oviduct and are negligible during the time course of the culture. BOEC responded to a stimulation with physiological doses of steroids, similarly to the in vivo situation. This in vitro model is therefore suitable to investigate the effects of various stimulants and can be employed for co-culture experiments with embryos to unravel details of the contribution of the bovine oviduct in embryo-maternal communication. This study was supported by DFG (FOR 478).

P24

Short term effects on pro-inflammatory cytokine, lactoferrin, and CD14 mRNA expression levels in bovine immuno-separated milk and blood cells treated by LPS.

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Leucocytes (WBC) are recruited from peripheral blood into milk as part of the inflammatory response, mediated through cytokines or interleukins synthesized by mammary tissue and the milk somatic cells (SC). The inflammatory response is related to the concentration of SC and the cytokines produced. To investigate and to compare the kinetics of cytokine production in SC and WBC during inflammation, cell culture models were established, where SC and WBC were cultured in parallel (n = 3). In addition, macrophages or monocytes were isolated from milk and blood with antibody-coated magnetic beads and cultivated separately. Isolated cells were pure, unaltered and viable. Cultures were activated with 10 µg/ml LPS. After 0, 1, 2, 3, 4 and 8 h cells were harvested for RNA isolation. Cytokine (TNFa, IL-1b, IL-6) mRNA expression responses and transcriptional activity of CD14 and lactoferrin (LF) were quantified via a one-step real-time RT-PCR. Significant cytokine mRNA increases were found in all four cell culture types and genes, with peaks after 1 and 2 h (TNFa > IL-6 > IL-1b). In WBC or monocytes higher LPS responses and longer persistence could be found than in corresponding milk cells (IL-1b > IL-6 > TNFa). SC and macrophages are less responsive to LPS stimulation than WBC or monocytes. The strength of the immune response in the blood system is much more prominent than in the mammary gland. This may be ascribed to the role of CD14 on the cytokine production of the investigated cells, or may be caused by the blood-to-milk diapedesis. The constitutive transcription of CD14 mRNA in WBC and monocytes was found to be 6- to 15-fold higher than in adequate milk cells.

P25

Expression of bovine ABC transporters - potential role of ABCA1, ABCG5 and ABCG8 in cholesterol transport in the mammary gland.

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BACKGROUND: Most of the known functions of eukaryotic ATP-binding cassette (ABC) transporters involve the shuttling of hydrophobic compounds either within the cell as part of a metabolic process or outside the cell for transport to other organs, or secretion from the body. Whereas ABC-transporters play a considerable role in hereditary human diseases, only scarce information is available about their expression and function in farm animals. Thus, we analysed the mRNA expression of ABCA1, ABCG5 and ABCG8, candidate ABC transporters involved in lipid metabolism, in bovine tissues.
MATERIAL AND METHODS: Total RNA from 18 different bovine tissues was extracted using the RNeasy Midi Kit (Invitrogen) and reversely transcribed into cDNA with SuperScript III (Invitrogen). Quantitative real-time PCR with gene specific primers was performed for the quantification of ABCA1, ABCG5, ABCG8 using LightCycler® technology. In addition, ubiquitin, GAPDH, 18S and β-actin were measured as housekeeping genes to normalize the data. Amplified products underwent melting curve analysis after the last cycle to specify the integrity of amplification. All runs included a negative control consisting of PCR-grade water; each sample was measured in duplicates. The relative quantification in the diverse tissues was calculated using bovine liver as reference. Data were analysed using the 2nd Derivate Maximum calculation described in the LightCycler® Relative Quantification Software.
RESULTS: We identified diverse bovine tissues with transcriptional activity for ABCA1, ABCG5 and ABCG8. These tissues are mainly involved in secretory function (mammary gland), metabolic function (liver), barrier function (lung, intestine), and trophic function (placenta, uterus). ABCA1 was amplified in all bovine tissues measured. High expression of ABCA1 was found in oesophagus and jejunum, in tongue, lung, heart, liver, spleen, uterus and muscle. ABCG5 and ABCG8 expression was found in abomasus, jejunum and colon, in liver, in the lymph nodes of jejunum, in mammary gland, blood and placenta. Both genes showed very high expression in colon and liver.
CONCLUSIONS: In concordance with other mammalian species, ubiquitous expression of ABCA1 and high expression of ABCG5 and ABCG8 in samples from liver and digestive tract was found. However important data arises from their expression in the mammary gland, opening new avenues for elucidating a potential role of these genes in cholesterol homeostasis and milk lipid secretion.

P26

Effects of Anabolic Sex Hormones on Gene Expression in Bovine Liver – Differential Gene Expression Profiling via a candidate gene approach qRT-PCR.

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The effects of anabolic sex hormones play a significant role in the establishment of muscle tissue. Therefore anabolic steroids have been used in animal production management schemes for several years to increase growth rate and feed efficiency. In the EU the use of hormonal growth promoting substances has been banned since 1988, because used in cattle they might possess a potential health risk to consumers. In other countries, e.g. USA and Canada, six hormones are allowed as growth promoters: zeranol, trenbolone acetate (TBA), melengestrol acetate (MGA), 17beta-oestradiol, progesterone and testosterone.

For this study 24 heifers were separated into three groups of eight heifers and were treated over eight weeks with multiple-dosages (0-, 1-, 3- and 10-fold) of either MGA (Melengestrol acetate, synthetic progestin), Ralgro (Zeranol, synthetic oestrogen) or Finaplex (Trenbolone, synthetic androgen). In each study two animals served as control, two received single, two 3-fold and two 10-fold dose of the recommended preparation. After selecting an assortment of anabolic sex hormone dependent genes from different biological functional groups (endocrine, adipolysis, oncogene, protein and amino acid metabolism, inflammatory, apoptosis or transcription) in the bovine liver, measurements of expression patterns were undertaken by quantitative real-time polymerase chain reaction (qRT-PCR). Aim of this study is to find treatment specific differentially regulated target genes, which can be used for further more detailed investigations.
Out of 24 examined genes, 6 genes showed a significant positive or negative regulation ($p<0.05$) under the influence of MGA, 6 genes under the influence of Finaplex, 3 genes under the influence of Ralgro, 3 genes under the influence of two different treatments either MGA or Finaplex and 2 genes under the influence of two different treatments either Finaplex or Ralgro. Only 4 of the observed genes remained unaffected by any of these treatments. The number of examined regulations confirm the used approach in selecting specific genes from functional groups which are associated with an anabolic sex hormone treatment. The study gives a first impression about the role of possible candidate genes and its expression patterns in drug screening sorted by its functional groups, with the goal to be used for veterinary drug screening at the level of toxicological relevance. After these examinations it seems interesting for the future to follow this candidate gene approach and to add more well-characterized genes to the functional groups and even extend the group selection.

P27

IGF-1 IN BOVINE SERUM AND LEUKOCYTES.

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During postnatal growth, IGF-1 stimulates protein synthesis and improves glucose utilization. As regulatory protein, it plays important role in the immune system by modulating immune responses, including lymphocyte activation e.t.c. Its binding proteins and receptors modulate IGF-1 biological activity. The aim of study was to investigate the expression of IGF-1 and IGF-1R in cattle leukocytes in early development. Whole (EDTA) blood and serum samples of 55 clinically healthy calves in age of four to six weeks were collected in Vorbuse and Rahinge farms. IGF-1 total content in blood serum was measured using RIA. mRNA was extracted from freshly stabilized blood samples using Roche mRNA Isolation kit. IGF-1 and IGF-1R mRNAs were multiplied using one step RT-PCR procedure in LightCycler with SYBR Green I detection. GAPDH and ubiqitine genes were used for relative quantification as housekeeping genes. The primers for RT-PCR (primer sequences obtained from publications of Pfaffl) were delivered by TIB MolBiol. GAPDH gene was more suitable for use as reference in cattle leukocytes. IGF-1 mRNA relative content in calve leukocytes vary in great range as well as the total IGF-1 content in blood serum (0.6 – 287 mg/l). However, there was low significant correlation between IGF-1 mRNA in leukocytes and IGF-1 content in serum. IGF-1R mRNA was in detectable amounts present in some cattle samples only. Our first results show that leukocytes may contribute to blood serum IGF-1 content, but the absence of IGF-1R mRNA shows no reverse effect. ESF Grant No 5734 supported this work.

Session

Poster Session: Normalization

Time: 5th Sep 2005, 18:30:00 - 5th Sep 2005, 20:00:00
Session Chair: Prof. Dr. Heinrich H.D. Meyer
Location: Student Cafeteria

P28

Novel reference genes for normalization of real-time PCR data in normal human tissues and an application to gene expression profiling.

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Genes with relatively stable expression across various tissues and experimental conditions can serve as promising candidates for normalization purposes in relative quantification studies using real-time PCR. We have established a human cDNA panel consisting of 12 neural (parietal, frontal, temporal lobe, thalamus, striatum, hippocampus, hypothalamus, substantia nigra, nucleus accumbens, cerebellum, spinal cord, dorsal root ganglia) and 12 peripheral tissues (liver, lung, heart, pancreas, adrenal gland, small intestine, spleen, skeletal muscle, kidney, bladder, stomach and thyroid). Pooled RNA samples derived from at least 4 donors were used for cDNA synthesis, followed by quantitative real-time PCR with SYBR Green chemistry to find the best reference genes out of ten candidates. These candidates were selected on the basis of minimal variation of their expression across human tissues, described in a public microarray database (HuGE database, Haverty et al., NAR, Jan 2002; 30: 214 – 217). Our results show that some of the frequently used “housekeeping genes” are not suitable for normalisation, since their expression varies significantly relative to total RNA among the 24 human tissues examined (as much as 70 and 11-fold for cyclophilin and beta-actin, respectively). We have identified three suitable reference genes, with relatively stable expression : G-protein pathway suppressor 1, proteasome subunit macropain beta type 2, and RNA-polymerase II, displaying a maximal variation between tissues of 6, 6.3 and 7-fold, respectively. In subsequent studies, we have normalised our data using the above three genes according to the method of Vandesompele et al (Genome Biology 2002, 3(7):research0034.1-0034.11). To demonstrate the utility of our human tissue panel, we have studied the gene expression pattern of three genes involved in amino acid metabolism and transport: D-amino-acid-oxidase (DAAO), D-Serin-transporter (Asc-1) and D-serine-racemase(DSeRac). We observed clear differences in their tissue distribution, as Asc-1 and DAAO is expressed mainly in CNS tissues, with DAAO reaching very high expression levels in the caudate nuclei and the spinal cord, while DseRac has comparable expression in the periphery and the CNS. Our tissue panel helps to define the gene expression pattern of putative drug targets, giving important clues to their roles in physiological and pathological processes, as well as shedding light to potential side effects.

P29

mRNA Expression of Apoptotic and Anti-apoptotic Factors during PGF2a Induced Luteolysis in Bovine Corpus Luteum.

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Apoptosis or programmed cell death is an important event in determining the lifespan and function of the Corpus luteum (CL) in several species. It is well established that apoptosis plays a critical role in structural regression of the CL. The aim of this study was to specify the expression of various apoptotic and anti-apoptotic factor family members in the bovine CL during PGF2alpha (PG) induced luteolysis. Cows in the mid-luteal phase (days 8-12) were injected with the PG-analogue Cloprostenol, and CL were collected by transvaginal ovariectomy before and 0.5, 2, 4, 12, 48 and 64 h after PG-injection. The mRNA expression was detected by quantitative real time PCR (Rotor Gene 3000). Investigated genes were the ligands of the extrinsic apoptotic pathway and their receptors (FAS-L and FAS antigen), tumor necrosis factor alpha (TNFalpha), its receptor (TNFR1). As representatives of the mitochondrial pathway BAX, BCL-XL, p53, Smac/Diablo and Survivin and three central caspases (Caspase3, -6, -7) were investigated.
The FAS mRNA expression showed a constant up-regulation to control from 2h on with the highest expression at 64h. For FAS-L all time

points were significantly increased to control level by being constantly expressed. TNF-R1 showed no significant regulation during induced luteolysis, whereas the expression of TNFa increased at all time points to control with the highest level at 2h after PG. BAX showed a down-regulation at 2h and then increased steadily to an up-regulated level from 24h to 64h. BCL-XL continued in the same way like BAX, however an up-regulation was first seen at 48h. An increase in expression level for p53 was revealed from 48h to 64h. Smac/Diablo was down regulated at 12h and showed an increase to control level at 64h. A decreased expression level of Survivin was recognised at 2h and 12h whereas, all other time points were not significantly regulated in relation to control group. Expressions of Caspase3 and Caspase6 indicated a constant up-regulation, which was significant from 12h on with the highest expression at 64h. Caspase7 was increased from 48h to 64h with a strong up-regulation at 64h. These results suggest, that apoptotic and antiapoptotic factors may play an important role in functional and structural luteolysis in the bovine CL. The first apoptotic signal seems to be received through the extrinsic pathway and may trigger the activation of the Caspases. The intrinsic pathway joins these activation 24h later when the structural regression initiates.
Supported by the DFG BE 3189/1-3 and Scha 257/14-2

P30

Mutations Induced at the Promotor Reguion of the myc gene due to Dual exposure to ionizing radiation and N-nitroso-urea.

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Malignant tumors arise from a sequence of events including mutations in protooncogenes and tumor suppressor genes. The accretion of these mutations is apparently facilitated by acquired or inherited defects in "guardian" mechanisms that maintain the integrity of the cellular genome. The proto-oncogene c-MYC, which is frequently overexpressed in tumors, is at the center of a transcription factor network that regulates cellular proliferation, replicative potential, growth, differentiation, and apoptosis. Expression of c-MYC down-regulated during differentiation and is rapidly induced by a diverse catalog of mitogens including ionizing radiation and many alkylating agents. In this present study we assessed the dual exposure to methylnitrose urea and ionizing radiation. The induced effects were assessed histopathologically, biochemically and were correlated at the molecular level by assessing single strand conformation polymorphism. Results showed that Administration of MNU with radiation increase the point mutation to 60% in rat liver hepatocytes. SSCP analysis of the my c-1 gene in the liver of control rats have showed neither myc gene mutations nor histopathological abnormalities. Point mutations of c-myc gene were detected in 40% and 60% of liver of male rats injected with MNU after 2 and 7 weeks respectively following the imitation period respectively. Ionizing radiation alone induced 20 % and combined ionizing radiation and MNU induced 60 % point mutations after 2 weeks. Histopathological changes and liver function tests correlated with percentage of focal damage. These findings are controversary to the suggestion that that c-myc amplification is cell-specific within radiation-induced carcinomas and does not occur in epidermal cells proliferating in response to radiation exposure. Also it can be suggested that a mutation at the promotor of the c-myc gene is responsible for the over expression recognized due to exposure to chemical and physical agents. Also the decrease in parameters observed after 7 weeks of exposure suggests that the increase in myc gene nutation was a compensatory mechanism to the external induced stress.

P31

Locked Nucleic Acid (LNA) Single Nucleotide Polymorphism (SNP) Genotype Analysis and Validation Using Real-Time PCR.

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In this study, we have used SNP specific LNA hybridization probes on the Rotor-Gene to genotype an association cohort and propose three criteria to address ambiguous genotypes. Based on the kinetic properties of PCR amplification, the three criteria address PCR amplification efficiency, the net fluorescent difference between maximal and minimal fluorescent signals and the beginning of the exponential growth phase of the reaction.
Criterion A: PCR amplification efficiency and genotyping is to be greater than 50% (E > 0.5) (E = 2/b) with a 5% error allowed for the weaker of the two fluorophore channels to compensate any preferential amplification. Criterion B: Net fluorescence gain is to be greater than 25% (DR > 0.25) x (DRx=a/y0). A 25% increase in fluorescent signal from the initial single copy of template DNA eliminates possible increases in fluorescent signal due to background fluorescent noise, non-specific binding and/or unincorporated PCR reagents. Criterion C: The difference in the estimated start cycle number between the two fluorophore channels must be less than two. If Criteria A and B are met for both fluorophore channels and Criterion C is satisfied, then the individual sample is truly heterozygous. If Criteria A and B are met for only one of the fluorophore channels and Criterion C is not met, then the individual sample is truly homozygous for the successful fluorophore channel.
SNP Identification: The investigated SNP was a C to T transition previously identified within the tryptophan hydroxylase (TPH) gene.
Real-Time Amplification: Real-time PCR was performed using the Rotor-Gene 3000 multiplex system (Corbett Research) in 25uL reaction volumes of, 200nM each PCR primer, 100nM each LNA probe, 1X MasterAmp PCR PreMix I (Epicentre(R)), 1.0 U Taq polymerase and 24ng genomic DNA. Cycling conditions were: initial denature at 95oC for 4min and then 45 cycles of 95°C for 15sec and 60°C for 45sec.
Sequencing: A number of genomic DNA samples (n=50) were sequenced to validate observed real-time PCR allelic discrimination curves and genotypes.
Association Cohort: Genotype data was obtained from a migraine Caucasian association cohort of 275 migraineurs and 275 aged and sex matched controls. From a total cohort (n=550), 131 (23.8%) individual genotypes were considered ambiguous. Of the 131 ambiguous genotypes, six (4.6%) samples differed in their calculated genotypes at the 5% and 1% error thresholds. These six samples were sequenced, of which five (83.3%) confirmed the application of the criteria at 95% confidence level. By applying a four-parametric sigmoidal model to raw fluorescent data, we have established three criteria to assess the validity and accuracy TM of possible ambiguous genotypes arising from the Rotor-Gene 3000 software.

Session

Poster Session: Optimization

Time: 5th Sep 2005, 18:30:00 - 5th Sep 2005, 20:00:00
Session Chair: Prof. Dr. Heinrich H.D. Meyer
Location: Student Cafeteria

P32

New information on detection of Plum pox potyvirus using real time PCR.

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Sharka, caused by the Plum pox potyvirus (PPV), is the most devastating viral disease of stone fruits and has a quarantine status in European Union and in Slovenia. First symptoms of sharka were observed in Slovenia in 1987 in several Prunus orchards. The virus is irregularly distributed in the plant so successful detection depends on sampling, the organ sampled and time of the year. At high temperatures the virus concentration in plants decreases and detection is less reliable. The real-time assay using MGB probe was developed for the detection of PPV in Prunus hosts.
The aim of our study was to determine the best method for total RNA isolation which would be appropriate for sensitive and reproducible PPV detection in routine diagnosis. Different commercial kits (RNeasy

Plant Mini Kit (Qiagen), SW Total RNA Isolation System (Promega), NucleoSpin RNA Plant (Macherey-Nagel)) were used for total RNA isolation according to manufacturer's instructions and/or with minor modifications. Quality and quantity of isolated total RNA was determined. Isolated RNA was used in subsequent two step RT-PCR reaction. Serial dilutions of cDNA were analysed and results for different isolations were compared. 18S RNA assay (Applied Biosystems) was used as internal control. Preliminary results in PCR based techniques show considerable differences in applicability between compared kits.

P33

Analysis of best primer and reverse transcriptase combination for the RT reaction using low amounts of bovine oocyte total RNA.

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Quantitative PCR (qPCR) analysis of gene expression in single bovine oocytes or early preimplantation embryos needs to meet optimized requirements for the isolation of total RNA, reverse transcription (RT) reaction and qPCR. Even though many studies have focused in this topic, there is a lack reliable protocol for RT reaction using such biological material. The objective of the current study was to test the optimal primer, reverse transcriptase and duration of the RT process. For all the experiments total RNA was isolated using a modified TriZol protocol (Boelhauve et al., 2005, BOR, in press) out of a pool of ten oocytes. An aliquot corresponding to one oocyte total RNA was reversed transcripted and qPCR was performed in an ABI PRISM 7000 SDS apparatus (Applied Biosystems) with SYBR-Green (Invitrogen) as a double stranded DNA-specific fluorescent dye. An aliquot corresponding to 10% of one oocyte was quantitative analyzed per reaction. All samples were performed in triplicates. Three gene transcripts were analyzed for the interpretation of the experiments: Signal transducer and activator of transcription factor 3 *STAT3* as a member of high-expressed genes, Histone 2a *H2AFZ* middle-expressed gene) and leptin receptor *LEPR* low-expressed gene). In the first experiment, the following primers were inserted with Superscript-2 reverse transcriptase (Invitrogen) in the RT reaction: random hexamer primer (Invitrogen and Applied Biosystems), random decamer primer (Ambion), oligo dT12-18 (Invitrogen), oligo dT16 (Applied Biosystems) and oligo dT18 (Ambion) as recommended by the manufacturers. This experiment was replicated 9 times. In the second experiment the best primer was tested with six reverse transcriptases: SuperScript-2, SuperScript-3, M-MLV (all from Invitrogen), RevertAid H- M-MLV (Fermentas), and SensiScript (Qiagen) in the RT reaction as recommended by the manufacturers. This experiment was replicated 8 times. In the last experiment, the best primer and reverse transcriptase were used to determine the best reaction time of the RT reaction. The reactions were stopped after 30, 60, 90, 120, 150 and 180 minutes. This experiment was replicated 8 times. The random hexamer primer (Invitrogen) was considered the best primer for these low amounts of total RNA since the transcript detection level was at least 4-fold higher ($p < 0.001$) than the other primers. The analysis of the best reverse transcriptase indicated that M-MLV-RT resulted in higher expression levels for high ($p < 0.05$) and middle ($p < 0.05$) amounts of gene transcripts. For low expressed genes Superscript-2 ($p < 0.05$) and -3 ($p < 0.05$) were the more optimal choice. The best duration of the RT reaction was observed after at least 150 minutes. The detection level rose up starting with 30 minutes and reached a plateau phase after 120 minutes for STAT3 ($p < 0.001$) and H2AFZ ($p < 0.05$) and 150 minutes for LEPR ($p < 0.001$).

P34

Design and validation of a robust diagnostic assay (prv-1 gene) based on real-time RT-PCR.

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Quantitative PCR of the prv-1 mRNA was set up and validated as a robust and reliable method for clinical routine diagnostics of Polycythemia vera rubra (PV) on the basis of peripheral blood specimens. Initially, the conditions of blood storage and shipping were evaluated. Then an optimised technique for cDNA preparation from granulocytes was established, followed by the identification of a panel of three reference genes out of a panel of six candidate reference genes, among them genes that had been suggested previously for hematologic diseases by the Europe Against Cancer group (EAC). This primary selection identified beta actin and G6PD as optimum reference genes that are clearly superior to B2M, GUS or ABL. The ranking of GAPDH remains controversial and depends on the mathematical model used for evaluation. On the basis of subsequent stability data where blood was stored at diverse ambient temperature the expression GAPDH proved to be rather unstable upon storage. On the basis of these data an assay was designed and validated. Limit of detection, limit of quantification, intra assay variability and inter assay variability were evaluated, respectively. Finally, a schedule for the collection of long term stability data was set up. Presently this assay is being clinically validated in a prospective trial. The modus operandi outlined here we suggest as a standard procedure for designing diagnostic assays based on quantitative real-time PCR.

P35

Simple Qualitative and Quantitative Methods for Measuring Residual Activity of "Hot-Start" Enzyme Preparations.

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PCR precision, specificity, sensitivity and yield of "Hot-Start" DNA polymerases are often directly related to the effectiveness of the method used to impart the "Hot Start" feature as well as the stability of the resulting product. For example, "Hot-Start" enzymes containing elevated levels of residual (not rendered dormant by chemical modification) polymerase activity (RPA) have been implicated in failures in some low copy and multiplex assays. Standard methods for quantifying RPA present in "Hot-Start" DNA polymerases or "Hot-Start" DNA polymerase Master Mixes involve tedious and time-consuming protocols based on direct measurement of P_32_ dNTP incorporation. Measuring fractions of a percent of residual activity in a Master Mix formulation is rather challenging, given the dilution of enzyme, even with a radiolabel. We have developed a set of non-radioactive, PCR-based assays useful for the detection and quantification of a wide range of RPA levels present in "hot start" DNA polymerase preparations and Master Mixes.
One-hundred fifty gene expression assays were screened for sensitivity to AmpliTaq Gold RPA by performing standard TaqMan reactions prepared with Master Mixes containing either AmpliTaq DNA polymerase or AmpliTaq Gold and comparing agarose gel electrophoresis band patterns. Assays demonstrating higher amplicon yields with diminished side products due to the "Hot-Start" were further characterized by titrating Taq Gold Universal PCR Master Mix (AmpliTaq Gold) with increasing amounts of AmpliTaq. Three assays showed clear-cut gel patterns that are useful for estimating incremental RPA in the 0-3%, 0-10% and 5-100% ranges. Another assay demonstrated high incremental sensitivity to RPA in the 0-1% range. Sybr Green PCR end-point melting curve analyses of 0% RPA (no added AmpliTaq) samples showed a major amplicon peak (Tm=81C) and minor dimer peak (Tm=74C). At 1% Taq addition the dimer peak increased while the amplicon peak completely disappeared. This phenomenon was exploited to develop a highly reproducible, quantitative Sybr Green assay method for RPA detection in the 0-1% range that can be performed on Applied Biosystems Sequence Detection platforms. The semi-quantitative gel assays and quantitative Sybr Green method both use standard "off the shelf" Applied Biosystems consumables and should be useful for PCR assay troubleshooting.

P36

Distribution of mRNA transcripts in single cells determined by quantitative RT-PCR.

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A cell contains approximately 20 pg of RNA, of which <5% is mRNA. That corresponds to a few hundred thousand transcripts, representing some 10,000 genes expressed at one timepoint. The constitution of this expression palette, or transcriptome, determines the fate of the cell and is a record of its recent history. Gene expression is ultimately controlled at the single cell level, but still, most gene expression analysis studies of today are carried out using thousands or millions of cells, for practical reasons. The measurements become a representation of the average cell, and individual differences in transcript levels remain undisclosed. Differences in a small proportion of the cell population are not likely to be revealed when looking at whole cultures or tissues.
When a small number of molecules determine the fate of a chemical equilibrium, a certain randomness and stochasticity is observed. As the number of molecules increase as do the predictability of the reaction. The number of enhancer and transcription activator molecules in a cell is low, and a stochastic element is thus seen in gene expression analysis at the single cell level. It has been suggested that some genes are expressed in a binary, on or off, behavior, resulting in a binomial population distribution of the transcript levels.
We have studied the gene expression of single cells in the pancreatic islets of Langerhans in mice using quantitative RT-PCR. The pancreatic islets are heterogeneous clusters of cells releasing major metabolic hormones, such as insulin. Precise quantification at this level has never before been carried out in tissues and data reveal intricate correlation between related genes while simultaneously showing a large spread between cells of the same type. Furthermore, we see a lognormal distribution of transcript levels in the single cell.

P37

A single-tube PCR with MGB Eclipse probes for detection of SHV-type extended-spectrum beta-lactamases (ESBLs).

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Objectives: ESBLs of the SHV-type are one of the most common and clinically significant beta-lactamases. The number of SHV variants is continuously growing however ESBL activity of SHV enzymes has been associated with mutations at relatively few amino-acid positions (aa-s) as compared to the TEM enzymes. Here we propose a simple and rapid method that allows detection of all the known SHV ESBLs in a single real-time PCR reaction.
Methods: The proposed method is based on amplification of blaSHV genes in the presence of short (13-14nt) fluorogenic probes capable of hybridization-triggered fluorescence. These probes commercially known as MGB Eclipse probes contain a dark quencher with a conjugated minor groove binder at the 5'-end and a fluorescent dye at the 3'-end. This structure allows detection and differentiation of nucleotide polymorphisms at targeted sites by post-PCR melting curve analysis. Four probes were designed to perfectly match the wild-type (WT) sequences at mutation sites corresponding to aa-s 146, 149, 156, 179 and 238. Thus, mutations conferring ESBL activity were expected to specifically lower the melting temperatures (Tm-s) of the probe-template duplexes. Each probe was labeled with a unique dye permitting analysis of mutations at multiple sites in a single reaction.
Results: The method was validated using laboratory strains producing the SHV-1 (WT, non-ESBL control), SHV-2, 3, 4, 5 (G238S), SHV-18 (G238A), SHV-6 (D179A), SHV-8 (D179N) and strains carrying cloned blaSHV fragments to which the naturally-occurring mutations D179G, G156D, T149S and A146V were introduced by site-directed mutagenesis. Following careful design of the probes and optimization of PCR conditions, all the above mutations were successfully detected and discriminated from the WT sequence and each other according to specific Tm-s. The detection was precise and highly reproducible in repeated experiments. Furthermore, when applied to the analysis of ten clinical isolates of Klebsiella pneumoniae expressing ESBL phenotype, the method was able to detect multiple SHV alleles (WT and G238S or D179A) in the same isolates. This observation is particularly important considering the high frequency of co-production of the SHV-1 and ESBLs in klebsiellae.
Conclusions: A PCR with MGB Eclipse probes has a great potential for studying the epidemiology of SHV ESBLs and possibly for analysis of other antimicrobial resistance mechanisms associated with mutations at defined loci.

Session

Poster Session: Standardization

Time: 5th Sep 2005, 18:30:00 - 5th Sep 2005, 20:00:00
Session Chair: Prof. Dr. Heinrich H.D. Meyer
Location: Student Cafeteria

P38

Association of Biomolecular Resource Facilities (ABRF) Nucleic Acid Research Group Study: A Comparison of Real-Time RT-PCR Technique, Chemistries and Hardware in Laboratories Utilizing the Same Assay.

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The Nucleic Acids Research Group (NARG) study for 2004-05 invited participants to run two real-time PCR benchmark tests utilizing an *in vitro* transcribed RNA or a synthetic DNA template for a human b-Actin assay. The templates, primers and probe and a diluent for the templates were provided by the NARG. The experiment entailed making separate 6-log dilution series of the RNA and DNA templates and generating a standard curve for each using the reagents provided. Each laboratory had the choice of running the assays using either Taqman® or SYBR® Green chemistry and the reagents and real-time instrumentation commonly used in their laboratory. The goals of this study were: 1) to provide the participants with feedback about their real-time PCR technique as measured by comparing the slopes, Ct values, r2 and y-intercepts obtained from the two standard curves generated from a DNA verses a RNA template; 2) to compare how the participants analyzed their data, e.g., baseline and threshold settings; 3) to gain information on how the various real-time instruments compare in their ability to detect signals over a 6-log range of template, and 4) to compare how the different real-time PCR chemistries perform using a standardized assay system. The results of this study will provide positive feedback for the participating laboratories and valuable information on the reagents and instruments available to the real-time PCR community.

P39

Development of a generic inhibition control for real-time PCR assays.

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In recent years real-time PCR has emerged as the main method used for DNA quantification. However, to ensure accuracy and precision with this technique it is essential that the analysis process is carefully controlled. For example, the performance of each stage of the testing process may be affected by substances present in the sample. A positive control sample can be used to monitor variability in test performance and can also be used to correct a quantified result for an amount of inhibition. Control samples are traditionally prepared by producing a concentrated sample of DNA, measuring the concentration of the DNA with a spectrophotometer and then diluting and aliquoting the DNA solution to obtain samples that contain very small amounts of DNA. Unfortunately, these methods do not yield reproducible controls samples. Significant errors are introduced by the dilution and aliquoting process due to the stochastic effects associated with pipetting low concentration of a DNA sample.
BTF Pty Ltd holds a propriety technology for the production of freeze dried BioBalls™ for delivery of precise amount of viable bacterial cells (patent WO 03/020959). It uses flow cytometry to dispense a precise number of cells into a droplet of fluid, which is then frozen and freeze dried. BTF's technology is being adapted to develop a generic inhibition control for real time PCR assays. The method involves generation of chromosomally-tagged bacterial strains and dispensing a precise numbers of cells carrying a known number of copies of a specific DNA sequence into freeze dried balls. The standard components of freeze dried balls, optimised to preserve the viability of the cells, were found to inhibit PCR assays. Alternative formulations for the freeze dried balls have been tested and optimised so that the ball matrix does not significantly inhibit PCR assays. The 'PCR-optimised' ball formulation produced freeze dried balls with a reasonably robust structure and utilises materials that are not likely to introduce foreign DNA or nucleases. This non-inhibitory matrix has been used to produce freeze dried balls carrying defined quanta of DNA for the development of a generic inhibition control for real time PCR assays.

P40

Expression of Active BCR Related (ABR) gene in Chronic Myeloid Leukemia (CML).

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Chronic myeloid leukemia (CML) is a clonal proliferative disorder of the haemopoietic stem cell cytogenetically characterized by the Philadelphia (Ph) chromosome, a result of chromosomal translocation t(9;22)(q34;q11). At the molecular level, the Ph chromosome results in a fusion gene, the chimerical *BCR-ABL* which has constitutive tyrosine kinase activity and is detected in virtually all cases at diagnosis. Indeed, the *BCR-ABL* gene expression has a pivotal role in the known pathogenetic mechanisms in CML cell proliferation and disease progression. Conversely, *BCR-ABL* inhibition with imatinib mesilate efficiently produces disease remission, since it is capable of selectively block the protein through occupying its ATP binding site. However, resistance to imatinib mesilate do occur and, although acquired mutations in the tyrosine kinase domain of *BCR-ABL* have been described, it seems that the appearance of acquired mutations, which result in gain of function, does not suffice for the resistant phenotype. Active *BCR* Related *ABR* gene is similar to *BCR* and both have a GTPase-activating protein (GAP) domain. Increased *ABR* activity has been detected in different solid tumors and more recently we detected over-expression of *ABR* in CML cDNA (EST) library.
The aim of this study was to investigate the expression levels of the *ABR* gene in peripheral blood and/or bone marrow samples of CML patients with real-time quantitative RT-PCR in different time points at diagnosis and after starting treatment. Fourteen CML patients were included and *BCR* beta-actin and *GAPDH* genes (geometric mean; GeNorm algorithm) were used as reference. Sensitivity dynamic range and efficiency were tested for all primers. Preliminary results showed that ABR gene expression was elevated at least five fold in CML samples at diagnosis. Currently, *BCR-ABL* expression is being detected and quantified for establishing a correlation between *ABR* expression levels and treatment with imatinibb mesilate in CML patients.

P41

Quantitative PCR assay set-up on the Eppendorf ep Motion 5070 using RealMasterMix Probe.

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The automation of diverse molecular biology methods has recently gained significance and is rapidly replacing classical manual protocols. Eppendorf has developed a product line that enables a streamlined and versatile approach to the automation of PCR-based applications. In the following experiments, Eppendorf automates quantitative PCR (qPCR) reaction set-up using the epMotion 5070 liquid handling station and RealMasterMix Probe, a reaction chemistry uniquely optimized for probe-based hydrolysis assays. The first experiment assesses the reproducibility of an automated reaction set-up. To determine well-to-well reproducibility, a 5-point standard curve with eight replicates for each point is created using a beta-2-microglobulin (B2M) probe and rat liver cDNA. To determine run-to-run reproducibility, this experiment is repeated three times each on two separate real-time PCR platforms, the ABI Prism 7000 SDS and the Bio-Rad iCycler iQ. These experiments are compared to three manual set-ups of the same experiment on the ABI Prism 7000 SDS. The next experiment demonstrates the efficiency of the epMotion 5070 in minimizing well-to-well contamination during reaction set-up when template is individually added to each well of a triplicate series. Fifty nanograms of genomic DNA template is alternated with water, transferred to the reaction plate, and cycled. Cross-well contamination did not occur during reaction set-up. Finally, in the Additional Applications Section, the epMotion 5075 is used to set up a similar 5- point standard curve using SYBR green and Eppendorf RealMasterMix, a reaction chemistry optimized for intercalation dyes and hybridization probes.

Session

Poster Session: BioInformatics

Time: 5th Sep 2005, 18:30:00 - 5th Sep 2005, 20:00:00
Session Chair: Prof. Dr. Heinrich H.D. Meyer
Location: Student Cafeteria

P42

Absolute Quantification in Real-time PCR by Nonlinear Regression Analysis - is the Standard Curve Obsolete?

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Background Absolute quantification by real-time PCR requires allocation of up to 1/8 of the wells of a 96 well plate for the standard curve. Furthermore, the cycle threshold method assumes equal PCR efficiency in all reactions, which is not always the case (e.g. in fecal samples). Previous attempts to generate alterative algorithms for absolute quantitation by curve fitting or regression analysis have shown variable success, and have been complicated to perform. Primarily, an alternative method should have a precision comparable to the cycle threshold (CT) method. Secondly, a simple hands-on procedure is necessary, as few have access to an experienced statistician on a daily basis. *Aim* To develop and evaluate an automated non-linear regression (NLR) algorithm capable of generating batch production regression analysis, with precision and characteristics comparable to the CT method. *Methods* Total RNA samples extracted from human gastric mucosa were reverse transcribed and analysed for TNF-A, IL4, IL18 and Beta-actin by TaqMan™ real-time PCR on an ABI-prism 7900 system. Fluorescence tracings were analysed by regular CT method with a standard curve, and by NLR with a positive control for optical calibration. Eleven separate regression models were tested, including combinations of log-transformation, weighted analysis, baseline correction, etc. Output data were subjected to Altman-Bland analysis. *Results* An automated algorithm was written in SPSS syntax. A 96 well plate could be analysed in less than 2 minutes. The Altman-Bland analysis showed that the best regression model had higher intra-assay variation, and a

mean bias of 43% (not significant). *Conclusions* The CT method is more precise for absolute quantification, but further development of the NLR algorithm may decrease intra assay variability. However, the versatility depends on the level of precision required, and in some settings, the increased cost effectiveness of NLR may justify a marginally lower precision. When inhibition is an issue, NLR may be more precise than CT, as PCR efficiency is included in the estimate.

P43

An Advanced, Web-Based, Software Program for Real-Time PCR Sequence Design.

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In a significant transformation of traditional PCR, real-time PCR reveals the target nucleic acid sequence through an accumulating fluorescent signal. Alongside this evolution, the design rules governing oligonucleotide sequence selection have also been refined with new insights and algorithms. We introduce a web-based software program, engineered to design both *TaqMan*® and *Amplifluor*® assays, that applies advanced computation toward the selection of primer and probe sequences. By fine-tuning a collection of parameters, the user can address primer-dimer formation, amplification efficiency, secondary structures, and mis-hybridizations. Interfacing with NCBI's databases facilitates sequence retrieval and specificity searches. Here, we demonstrate that this software program designs robust assays that correctly amplify their targets from a panel of human genes, confirming its role as a valuable tool for qPCR applications.

P44

AssayBank: A simple database for tracking QPCR assay performance for small-scale labs.

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Quality control is a valuable guide tool for assay performance in real-time QPCR. Even in small laboratories with a limited number of users, it is advantageous to track assay inventory, primer sequences, tissues tested for each assay, assay efficiency for runs and several other parameters. Most quality control software are large and expensive LIMS systems. We have instead developed a simple Filemaker (TM) database, AssayBank, to deal with small laboratory needs. Features of the database will be presented. The database may be run on a single computer or over a network using either Filemaker access or web browser (Internet Explorer 6 (TM)).

P45

REST 2005 - a new standalone software for efficiency-corrected relative quantification.

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REST 2005 is a new standalone software tool to estimate efficiency-corrected relative gene quantification results, while incorporating multiple sources of experimental variation. The algorithm addresses issues surrounding the measurement of non-linear variation from traditionally difficult distributions such as expression ratios and standard dilutions, by using randomization and bootstrapping techniques. Housekeeper normalization over multiple genes using the geometric mean provides further control over the experiment. The hypothesis test has been enhanced to incorporate variation in the efficiency measurement, and by increasing the number of iterations from 2,000 to a fixed 50,000, now achieving a level of consistency on par with traditional statistical tests. New confidence intervals for expression levels also allow scientists to measure not only the statistical significance of deviations, but also their likely magnitude, even in the presence of outliers. Expression ratio results are presented in a whisker box-plot, providing a visual representation of variation for each gene that highlights potential issues such as distribution skew.
http://rest.gene-quantification.info/

P46

Theoretical modeling qPCR with chemical thermodynamics.

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Quantitative PCR (qPCR), being one of the non-array gene expression quantification technologies, is getting more and more attention from the research community. This is partly because of the headaches that people had had in the application of DNA microarray: the unclear theoretical model, the algorithm in the data analysis, the inconsistency of data and the data analysis results. However when comes to quantification of mRNA (or DNA) by qPCR, the same problems will face us again. A theoretical model is definitely needed to describe what is going on and how the quantification is done through qPCR. This paper attempts to theoretically modeling the qPCR by chemical equilibrium and thermodynamics so as to supply a mathematical computation basis for the technology.

Session

Poster Session: GMO & Food Hygiene

Time: 5th Sep 2005, 18:30:00 - 5th Sep 2005, 20:00:00
Session Chair: Prof. Dr. Heinrich H.D. Meyer
Location: Student Cafeteria

P47

Gene expression analysis of enterohaemorrhagic *Escherichia coli* virulence using qRT-PCR assay.

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Enterohaemorrhagic *Escherichia coli* (EHEC) serotype O157:H7 colonizes the large intestine of humans, where it causes the attaching and effacing (AE) lesions. Once attached EHEC produces shiga toxins, which are responsible for the major symptoms of hemorrhagic colitis and hemolytic uremic syndrome. The genes involved in the AE lesion formation are encoded on the pathogenicity island known as the locus of enterocyte effacement. The expression of this pathogenicity island is controlled by the bacterial cell-to-cell signalling mechanism known as quorum sensing (QS). This signalling system is mediated by small molecules known as autoinducers (AI). EHEC produces AI-2, alike most AIs, which are species specific, AI-2 serves as a “universal” signal, allowing bacteria in the gastrointestinal tract to communicate. Probiotic bacteria often coexist with potential pathogens without establishment of symptomatic infection. This has been attributed to probiotic ability to inhibit or displace pathogens, which has been considered as a probable way of preventing infection. However, other important factors may be involved. The aim of the current study is to determine the effect of probiotic *Lactobacilli* on the expression of virulence in EHEC and their role in QS by means of RT-PCR.

P48

Direct quantification of pathogens in food samples by filtration and qPCR without the need for enrichment or DNA purification.

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QPCR has become an established diagnostic tool and research method in many different fields. Also in the area of food microbiology and the detection of pathogens in biological samples it has increasingly gained popularity. Although many different protocols have been set up, a few common challenges remain. The main limitation of this application currently is the small sample volume of 1-10 microliter that is used in qPCR analysis. For food sampling, often samples as large as 10 gram or 25 gram in 100 or 250 ml solution are generated. As the ideal goal is to detect 1 cell in such a sample, it is statistically much easier to detect that single cell, when the whole sample volume can be used for analysis. To date, only a very small number of studies have successfully used larger samples for direct detection of very low amounts of pathogens (between 10^1 – 10^2 CFU per gram sample) in food. Recently we have developed a new two-step filtration protocol followed by an absolute qPCR assay based on hybridization probes in order to directly quantify food-borne pathogens in several types of biological samples: such as chicken rinse and spent irrigation water. The whole procedure could be completed within 3 hours from sampling to detection, and cell numbers as low as 7.3 CFU/ 100 ml biological sample were detected. This study has thus made another step toward simple, rapid and reliable quantification of pathogens in food and environmental samples without the need of sample enrichment or DNA extraction. Currently, research is ongoing to investigate possibilities to combine the current method with virus-capturing filters for quantification of both bacterial and viral pathogens in biological samples.

P49

RAPID DETECTION OF *Campylobacter jejuni* BY IMMUNOMAGNETIC SEPARATION AND REAL-TIME qPCR.

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Campylobacter jejuni is a food-borne pathogen considered as the leading cause of acute bacterial diarrhea world-wide. It can be primarily found in a wide range of foods including poultry, pigs and beef. The traditional detection of this microorganism using culture-based methods is time-consuming, laborious and difficult to adapt for quantitative analysis (1). Therefore, it is necessary to implement new detection methods which present high reproducible sensitivity, marked specificity, and speed in obtaining results. Real-time qPCR is an alternative which enhance both productivity and analytical flexibility to detect pathogen in food. However, there is a possibility that food matrices could interfere with the fluorescence signal detection or with the DNA amplification of the real-time PCR system (2). A suitable alternative to solve this disadvantage is the pretreatment of the sample to avoid the reaction inhibition and to isolate the bacteria. Pathatrix is an immunomagnetic separation method which allows the isolation of cells from the food matrix. Furthermore, this system is the only microbial detection method able to process large sample volume (25g) by re-circulating the sample through a "capture phase". This study is focused on the detection improvement of Campylobacter jejuni in food samples using Pathatrix and real-time qPCR. Our preliminary results showed that changing some parameters in the Pathatrix system can refine the cell recovery. Besides, the use of real-time probes to confirm the Campylobacter presence speed the detection up. The use of both systems, Pathatrix and real-time qPCR seems a better option to detect Campylobacter jejuni in food samples.

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P50

Comparison of an IMS-real-time PCR-method with a cultural reference method for the detection of *Salmonella spp.* in meat.

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Conventional cultural methods for the detection of *Salmonella* spp. in foods are time-consuming. A preliminary result is achieved after five days and it takes seven days to get a confirmed result. For an accommodative consumer protection, methods are necessary that provide confirmed results in one day and the quality of these results accord with those of the reference method.
In this study an IMS-real-time PCR-method for the specific, sensitive and rapid detection of *Salmonella* spp. in foods was compared with the method L 00.00-20 of the Official Collection of Methods of Analysis according to §35 of the German Law on Food and Commodities. According to §35 the reference norm was given by the results gained after the Selenite-Cystine medium as well as the Rappaport-Vassiliadis medium had been incubated for 48 hours. The statistical analysis was carried out according to HÜBNER ET AL. (2002). The IMS-real-time PCR-method included a preenrichment step of 6 hours (37 °C), immunomagnetic separation, DNA extraction as well as DNA purification followed by a real-time PCR analysis. The efficiency of the rapid method was assessed with regard to the reference method analysing artificially and naturally contaminated meat samples. Both methods analysed the same preenrichment so an accurate comparability was achieved.
Compared with the reference method and regarding artificially contaminated meat samples (about 1 cfu/25 g; 10 cfu/25 g; 100 cfu/25 g and 0 cfu/25 g) the IMS-real-time PCR-method achieved a specificity of 80 % (false-positive rate of 20 %) and a sensitivity of 100 % (false-negative rate of 0 %). The relative accuracy was 94 %. The detection limit of both, the IMS-real-time PCR-method and the reference method, was 10 cfu/25 g. The concordance index kappa, that defines the statistical accordance showed 0,85 what indicates that the IMS-real-time PCR-method and the cultural reference method agree on statistical criteria. The efficiency of the IMS-real-time PCR-method compared to the reference method was also examined using naturally contaminated meat samples (n = 491). Compared to the reference method the IMS-real-time PCR-method showed a specificity of 99,3 % (false-positive rate of 0,7 %) and a sensitivity of 83,7 % (false-negative rate of 16,3 %). The relative accuracy was 98 %. The concordance index kappa had a value of 0,87 what indicates that both methods statistically agree.
References:
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P51

Quantification of GMOs in food: Assessment of measurement uncertainty (MU).

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Credibility of analytical data has never caught the public's eye more than today. Quality and comparability of measurement results require that these are reported together with their measurement uncertainty (MU). Presented is an approach for evaluating MU of quantitative real-time PCR data for GMOs. The approach is based on the classical, component-by-component or 'error-budget' approach, described in the ISO-GUM and Eurachem/CITAC-QUAM guides. It bases on the following steps: (1) specification of what is being measured, (2) identification of all sources of uncertainty, (3) quantification of standard uncertainties and (4) calculation of combined and expanded uncertainties. Data from intralaboratorium validation studies and internal quality control (IQC) measures were used for the estimation of four standard uncertainties: precision, bias, the uncertainty associated with reference materials (RMs) and sampling and sample preparation error. Mean MU values of 0.1 % and 2.5 % were obtained for GMO concentration levels of 0.1 % and 5 % respectively, in soybean and maize flour. These uncertainties should always be reported together with any quantitative result.

P52

Detection and quantification of genetically modified organisms (GMOs) in food.

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A universal and integrative analysis scheme for GMO detection, identification and quantification is presented. Raw products as well as solid and processed derived products can be analyzed: soybeans and soybean-derived products on the one hand, maize or corn seeds and maize-derived products on the other hand. The analysis is built up in a modular way. A specific sample preparation and a silica-based DNA extraction are followed by a PCR step. A decision support system is presented as a helpful tool for choosing the analysis scheme, which is reliant on the matrix under study and the specific analytical question. When setting up a general scheme for routine GMO analysis, it is important to offer different options. There is no single method or technique, covering all analytical questions and applicable for all matrices along the chain. An aspect that is determinative for the choice of strategy, is the cost price. The higher the number of events to be tested and/or quantified, the more heavily cost will weight. Working costs are reduced significantly as we use one and the same technique, real-time PCR, for different purposes. Real-time PCR benefits from the fact that amplification and detection are combined in a closed system, eliminating any post-PCR step and reducing both time and effort.
GMO analyses are carried out in function of implementation of the GMO legislation: Directive 2001/18/EC, Regulations (EC) 1829/2003 and 1830/2003 and Recommendation (EC) 2004/787. Monitoring the quality of analytical results is a key issue in the ISO/IEC 17025 standard, prescribing general requirements for the competence of testing and calibration laboratories. For this reason, we invested in the setting up of a quality laboratory for GMO analysis. Based on external audits carried out by Beltest, since June 2004, our laboratory is accredited for qualitative and quantitative GMO analyses on raw and processed products.

P53

GMO detection/quantification in different food matrices: not an easy task.

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European legislation requires labelling of GMOs in food and feed products in raw materials as well as in processed foods. The GMO laboratories are therefore faced with a wide spectrum of different samples from which DNA is extracted and analysed by PCR. The concentration of extracted DNA is often low and is limited especially in processed food which is subjected to high temperature treatments and acidity. Small amounts of isolated DNA are problematic due to false negative results and high LOD/LOQ for GMO presence/quantification. Additional problem is the presence of PCR inhibitors. Food products from different food producers differ in composition, which is often unknown to the analyst and there is a great variability in DNA content and quality within the same type of food matrix.
We have chosen different often analyzed soybean and maize samples that represent either raw plant material, feed or processed food matrices. The amount of DNA that extracted was compared. Samples were analysed by Real-time PCR and the effect of matrix on real-time PCR efficiency was assayed using sample dilutions. Samples that represent the problem regarding the inhibition were identified. Different effect on PCR efficiency was observed if analysing different amplicons as well as if the DNA was extracted using a different methods from the same matrix.
However, even the samples without significant inhibition during the qualitative analyse could represent the problem for quantification of GMO and many samples are quantified twice or even DNA had to be isolated with another method and quantification repeated to fulfil the criteria for quantification.

P54

Feeding genetically modified Bt176-maize – no effects on selected ruminal bacteria.

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Transgenic maize Bt-176 produces the crystal (Cry) protein to induce resistance against Lepidoptera, like the European Corn Borer *Ostrinia nubilalis* . However, scarce information is available about the effect of Cry-protein on rumen bacteria after feeding genetically modified maize. Therefore five rumen-fistulated cows were first fed with isogenic maize (Antares) for 41 days and thereafter with transgenic maize (Navares) for 16 days. Rumen content samples were taken and bacterial DNA was isolated using a commercially available kit. For quantitative determination of five representative rumen bacteria (_Anaerovibrio lipolyticus, Fibrobacter succinogenes, Prevotella ruminicola, Selenomonas ruminantium, Treponema bryantii_) the LightCycler™ system was applied. Final concentrations were calculated using the “standard curve method”; for data normalization 16S-rDNA was used. For every bacterial strain the real-time PCR results for all cows throughout the studies were summarized and the mean ratio ± standard deviation (SD) was determined. Furthermore, the interday and intraday coefficient of variation (CV) for repeated measurements of the rumen bacteria was determined. Standard curves of the LightCycler™ runs were linear within the measurement range and unique melting curves demonstrated that only the expected DNA-fragments were amplified. Runs on consecutive days did not yield significantly different results. The interday CV (n=3), analysed for two bacteria and 16S-rDNA, varied between between 0.67 and 1.62 %; the intraday CV (n=4), analysed for *Treponema bryantii* was 1.71 %. These results underline the accuracy of the real-time measurements. For *Anaerovibrio lipolyticus* a mean ratio of 1.03 ± 0.63 was observed. The amount of *Fibrobacter succinogenes* varied up to 6-fold (mean ratio 1.45 ± 0.81). A mean ratio of 1.58 ± 0.70 and 2.85 ± 1.66 was obtained for *Prevotella ruminicola* and *Selenomonas ruminantium* respectively. The levels of *Treponema bryantii* varied 1-fold over the course of the study; the mean ratio ± SD was 1.18 ± 0.65. A periodical increase in the amount of the five rumen bacteria was observed after feeding both isogenic and transgenic maize, which indicates that changes in the composition of the rumen microbial system cannot be associated with the feeding of genetically modified maize. Interindividual variations between the cows were even higher than differences observed after the diet shift from isogenic to transgenic maize.

P55

Confirmation and Quantification of Leptospiral Burden in a Human Population using Real-Time qPCR.

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A prospective, population-based study was carried out to identify febrile patients exposed to Leptospira in urban and rural contexts in Iquitos, Perú. Evidence of exposure to Leptospira was obtained by serology for patients. Leptospirosis was confirmed in cases with pulmonary involvement by demonstration of the organism by culture and/or on-site performance of a quantitative real time PCR assay. The results of the real-time qPCR assay showed high levels of leptospiremia in most fatal cases (>10^4 leptospires/mL); one patient from whom tissues were obtained at autopsy had >10^5 leptospires/g of lung, kidney, and muscle. This study demonstrates the benefits of a rapid, real-time qPCR assay to confirm the diagnosis of leptospirosis in a highly endemic region, including recognition of grave pulmonary complications.

P56

No Impact of feeding genetically modified maize on the immune system of cows.

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Background: In 2004 the worldwide area of transgenic crops has increased to 81 million ha. Those biotechnology-derived plants are predominantly used as feed for farm animals. Our work addressed the question, whether feeding of transgenic maize (Bt176) might have an effect on the immune system of dairy cows. Therefore, selected marker genes of the immune system were measured in various bovine tissues using real-time PCR.
Material and Methods: Total RNA from bovine tissues (liver, kidney, spleen, ruminal epithel, lymph node) of 2 cows fed with isogenic and 4 cows fed with transgenic maize was extracted using the Trifast®-method or the RNeasy Mini Kit® (Invitrogen). After a reverse transcription into cDNA, quantitative real-time PCR was carried out using LightCycler® technology. Gene specific primers were applied to amplify the immunomarker genes TNF-alpha;, interleukin-1beta (Il-1ß), and lactoferrin. For data normalization, GAPDH as housekeeping gene was used. Melting curve analyses and negative controls consisting of PCR-grade water assured specificity of the amplified products. Each sample was measured in duplicates. Data were analysed using the 2nd Derivate Maximum calculation described in the LightCycler® Relative Quantification Software.
Results: The expression measurements of all genes achieved better results for mRNA extracted with the Qiagen Rneasy Mini-Kit® in samples with a high content of connective tissue. Considerably higher expression of TNF-alpha; and Il-1 ß could be found in the lymph node and the spleen as compared to kidney, caecum, and rumen epithel. Lactoferrin expression was high in liver and caecum epithel, while low levels were found in kidney, rumen, and lymph node. No difference in cytokine (TNF-alpha, Il-1ß;) and lactoferrin expression was found between tissues of transgenic and isogenic fed dairy cows.
Conclusion: Real-time quantification of selected immunomarkers in different bovine tissues showed no differences between isogenic and transgenic fed cows. Our data suggest that the recombinant protein in Bt176 maize does not induce immunoactive processes in the bovine organism.

P57

In situ study on the time-dependent degradation of recombinant maize DNA in the bovine rumen.

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Genetically modified maize like event176 incorporates a truncated form of the *cry1Ab* gene from Bacillus thuringiensis (Bt) to protect itself against the European corn borer. Since the market release of genetically modified plants, questions regarding the digestive fate of biotechnology-derived DNA in farm animals have been raised. Thus, the objective of our work was to establish reliable real-time PCR methods for quantification of plant and recombinant DNA in order to monitor the ruminal degradation of DNA in genetically modified maize (whole plant and ensiled maize). For analysis of fed plant DNA, primers spanning a 199 bp fragment of the highly-abundant *rubisco* gene were optimized for LightCycler® technology. Melting curve analyses assured specificity of all amplified products. For quantification of the single-copy *cry1Ab* gene using LightCycler technology, specific primers covering a 110 bp region of the *cry1Ab* gene and two dye-labeld oligonucleotides were designed. To exclude possible unspecific amplification, isogenic maize samples of all time points were analysed in parallel. Results were compared to those obtained by a commercially available Bt176 maize quantification kit (Roche). Both for *rubisco* and *cry1Ab* gene quantification, negative controls consisting of water were included in all runs; each sample was run in duplicates. The results for both genes were expressed as percentages of respective initial values which were measured in samples without ruminal incubation.
Values of the *rubisco* gene in maize samples decreased to 20% after 2 h; by 4 h they were less than 6% of the initial values. After 48 h of ruminal incubation the values were less than 0.5% for both Bt- and non-Bt maize. Initial values for the *rubisco* gene measured in ensiled plant samples were approximately 0.9% of initial values found in whole plant maize samples. The degradation pattern of *rubisco* DNA did not show a difference between isogenic and transgenic maize samples.
Quantifying *cry1Ab* DNA showed a sharp decrease during the first 4 h of incubation to less than 1% of initial for whole plant maize samples, which ended up in final values of less than 0.5% after 48 h. The quantification of *cry1Ab* DNA in ensiled maize samples reached the detection limit after 8 h of ruminal incubation. Results after that time point were not reliably distinguishable from values of isogenic maize samples. However, the initial value of ensiled maize was only 0.62% of the initial value of whole plant transgenic maize. Very similar results were obtained for the commercially available kit and our own protocol. No obvious differences between the degradation pattern of *rubisco* DNA and *cry1Ab* DNA were observed. In summary, an extensive time-dependant degradation of plant and recombinant DNA in transgenic maize in the bovine rumen was demonstrated. Thus, it is very unlikely that transgenic DNA shows functional activity after exposure to the bovine rumen environment.

Session

Poster Session: Array Verification

Time: 5th Sep 2005, 18:30:00 - 5th Sep 2005, 20:00:00
Session Chair: Prof. Dr. Heinrich H.D. Meyer
Location: Student Cafeteria

P58

Validation of a bacterial 70mer oligo microarray using the Exiqon human probe library.

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Identification section (1) and department of Genomics & Strain Development(2), Chr. Hansen, Denmark.

The availability of ready-made assays or validated probe libraries for gene quantification is very dependant on the target organism. While these assays or libraries are abundant for human, mouse, and rat they are typically not available for bacteria. We can however demonstrate almost full coverage of a bacterial genome with the human probe library from Exiqon. The complete DNA sequence of the gram+ bacterium Lactococcus lactis IL1403 is publicly available. Based on this sequence a 70mer oligo microarray platform was set up in our laboratory for practically all the 2266 predicted genes. Gene expression data from the whole genome microarrays was compared to qPCR data on 42 selected genes. Assay design: The DNA sequences were submitted to ”Probelibrary Design Center” and 39 of the 42 genes (93%) could be covered by assays based on this library. Assays were performed on an ABI 7500 platform using ”Low ROX probe mastermix” from Eurogentec. The quality of the qPCR assays and the consistency with the whole genome arrays will be discussed.

P59

Genome Wide Expression Profiling of Paired Cancerous.

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Applied Biosystems, 850 Lincoln Centre Drive, Foster City, CA 94404.

In this study we profile over 30,000 genes in paired normal and cancerous breast tissues on the Applied Biosystems Expression Array System. By comparing paired breast tissue specimens from the same patient we can identify genes that are differentially expressed in normal tissue, primary tumor, and metastasis tumor. Both biological replicates and technical replicates have been included in this study. Using statistical data analysis tools, we have identified numerous genes that are up- or down-regulated in primary tumor and metastasis tumor compared to their normal control. Clear changes in gene expression patterns from normal to primary tumor and to metastasis tumor are observed. Gene expression level changes for genes

detected by the Applied Biosystems Expression Array System have been validated using quantitative real-time PCR (TaqManÒ probe-based Assays). This study demonstrates the use of microarrays for genome wide screening for gene expression in combination with quantitative real-time PCR for validating and extending results for genes of interest.

P60

Validation of a transcriptomics analysis using real-time qPCR: Deciphering embryo-maternal communication.

Susanne E. Ulbrich [1], C. Klein [2], S. Bauersachs (2, 3), R. Einspanier [4], E. Wolf (2, 3), H.H.D. Meyer [1] (ulbrich@wzw.tum.de)
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(3) Laboratory for Functional Genome Analysis (LAFUGA), Ludwig-Maximilian-University Munich, Munich, Germany
(4) Institute of Veterinary Biochemistry, Free University of Berlin, Berlin, Germany.

In an attempt to identify genes, which are presumably involved in embryo-maternal communication and induced in the bovine endometrium by the signaling of the early embryo, a transcriptomics approach was applied. A combination of subtracted cDNA libraries and cDNA array hybridization was used to compare endometrial tissue samples of day 18 pregnant and control animals. The present study aimed to verify the results of the array hybridizations using real-time RT-PCR, a highly sensitive and reliable mRNA quantification technique.
Five monozygotic twin pairs (Simmentaler Fleckvieh) generated by embryo splitting were cycle synchronized. At day 7 after standing heat two in vitro produced embryos were transferred into the ipsilateral uterine horn of one twin of each pair. The corresponding twin received a sham-transfer without embryos as a control. At day 18 the animals were slaughtered. The uterine lumen was opened longitudinally. Intercaruncular endometrial tissue samples from defined uterine regions, which had been in contact with an embryo were used and corresponding tissue in the non-gravid twin. A combination of subtracted cDNA libraries and cDNA array hybridization was applied to identify differentially expressed genes in the gravid compared to the non-gravid uterus. To validate cDNA array hybridization data mRNA concentrations of nine selected genes were relatively quantified by real-time RT-qPCR (LightCycler®). The same RNA samples were used allowing a direct comparison of both techniques.
The combination of SSH and microarray technique revealed 90 different up-regulated genes and mRNAs. A classification of the genes based on Gene Ontologies showed further the up-regulation of genes important for cell adhesion, cell differentiation, and cell communication. For all eight transcripts, RT-PCR results were in good correlation with the results obtained by array hybridization. Due to technical reasons of the hybridization for broad differences the array tends to underestimate transcript differences. Further comparative analyses will now follow in order to confirm the relevance of the results in a large number of individual samples as well as a in more detailed compartments of the uterus and other reproductive tissues. The differential gene expression obtained by the holistic transcriptomics approach was clearly validated and strengthened in more detail by qRT-PCR. A combination of both transcriptomic and candidate gene approach seems most promising to identify and study thoroughly mechanisms of embryo-maternal signaling.
This study was supported by DFG (FOR 478) Research Unit Mechanisms of embryo-maternal communication

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P61

ENGL - The European Network of GMO Laboratories.

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The European Network of GMO Laboratories (ENGL) was founded on the 4th of December 2000. At present 72 laboratories from 25 EU states plus Norway are officially registered ENGL members. The legal basis of the ENGL is Regulation (EC) No. 1829/2003 on genetically modified food and feed. All laboratories and delegates are nominated by the competent authorities of the member states and all these laboratories are assigned with official food/feed/seed control in the GMO sector. The ENGL is chaired by the Joint Research Centre (JRC) represented by its Institute of Health and Consumer Protection, Biotechnology & GMOs Unit. The work of the ENGL is supported by a Steering Committee (SC) which coordinates and prepares decisions of the ENGL. Each country is represented by one delegate in this SC.

The main tasks and activities of the ENGL are as follows:
- performing validation studies in the context of the GMO authorisation process
- supporting the Community Reference Laboratory (CRL) in its activities
- forming a scientific and technical European Union network in the context of EU GMO regulation
- contribute to the European standardisation and harmonisation of methods for sampling, detection, identification and quantification of genetically modified organisms and derived products covering seed, grains, food, feed and environmental samples
- defining performance criteria for GMO detection methods provided by applicants for GMO authorisation
- development of methods for qualitative and quantitative GMO analysis
- initiation of research programs
- working groups for sampling, threshold, validation, reference material, decision tree

The ENGL is involved in various projects (selection):
- ENGL Molecular Register: The ENGL Molecular Register is a collaboration with the National Research Council (CNR), Italy and the RKI, Germany. This molecular register shall contain all the molecular details of the EU-approved GMOs and the necessary on-line tools to analyse those sequences (restricted access). It is organised around a central database storing the GMO molecular details including elements on the associated detection method.
- ENGLnet: The ENGL member intranet (restricted access) shall serve as a collaboration tool for the exchange of common information among all ENGL members and members of the SC.
- Plasmid project: Establishing a collection of plasmids that contain cloned amplicons relevant for qualitative and quantitative GMO analysis in food and feed control. The plasmids shall serve as control samples independent of the availability of reference material produced from dried plant material.

There are five ENGL delegates of Germany:
- Hans-Jörg Buhk, RKI, Berlin, buhk@rki.de
- Hermann Broll, BfR, Berlin, h.broll@bfr.bund.de (SC)
- Norbert Hess, BWG, Hamburg, norbert.hess@bug.hamburg.de
- Sven Pecoraro, LGL, Munich sven.pecoraro@lgl.bayern.de
- Klaus Pietsch, CVUA, Freiburg, klaus.pietsch@cvuaf.bwl.de

Web: ENGL: http://engl.jrc.it/ ; CRL: http://gmo-crl.jrc.it

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qPCR Application Workshop

powered by TATAA Biocenter, Sweden
www.tataa.com

Freising 7-9th September 2005

Practical Room **P2**

This workshop is aimed at giving participants a deep and objective understanding of real-time quantitative PCR and its applications. The courses are intended for persons considering working with qPCR or scientists currently working with qPCR seeking a deeper understanding.

The course covers all aspects in qPCR, from sample preparation to data analysis and is held during 3 days. The course is approximately 50% hands-on as is limited to 20 participants, resulting in very interactive teaching and everybody given the opportunity to try the instrumentation.

Examples of topics covered in the workshop:

- Basic Principles of PCR and qPCR
- Comparison of different detection technologies
- Applications of qPCR
- Probe and Primer design
- Data Analysis
- Relative Quantification-considerations and limitations
- Experimental Design
- Reverse Transcription
- Extraction methods
- Multiplex considerations

After the course participants will be able to plan and perform qPCR experiments themselves, as well as interpret and analyze data.

TATAA qPCR Application Workshop is supported by the following companies:

Roche eppendorf

LTF Labortechnik corbett RESEARCH

Invitrogen life technologies BIO-RAD

EUROGENTEC EGT GROUP STRATAGENE

Preliminary Schedule for TATAA Biocenter qPCR workshop

Freising 7-9th September 2005

Practical Room **P2**

The course is focused on practical issues for qPCR and are partly hands-on, performed by the course participants in the lab (marked in blue). Preliminary agenda for download TATAA-qPCR-WS.pdf
Lunch, coffee and snacks are included in the course fee.

Wed. 7th September
Day 1 - Basic qPCR

13:00-14.00
Basic PCR and qPCR theory and applications

- Amplification and detection
- Detection chemistries
- Selected applications

14.00-15.00
qPCR experiment by participants

- Display of various instrument platforms
- Demonstration of qPCR software
- Practical considerations when preparing PCR reactions
- Programming qPCR machines

15.00-15.15
Coffee Break

15.15-16.00
Primer and probe design and considerations

- What does primer design affect?
- What are primer dimers?
- How do we minimize formation of primer dimers?
- Design of Molecular Beacons and TaqMan probes

16.00-17.00
Data analysis

- How does qPCR software process the data?
- How are standard curves used and created?
- How are melt curves used?
- Principle of quantification using standard curves
- Principle of relative quantification

17.00-17.30
Analysis of performed qPCR experiments

17.30-17.45
Discussion and Q&A

17.45
End of qPCR workshop day 1

Thu. 8th September

Day 2 - Advanced qPCR. Quantification, Normalization and experimental design

09.00-09.50
qPCR quantification strategies

- standard curves
- relative quantification
- how to compensate for inhibition in biological samples

09.50-10.15
Normalization of qPCR data

- What levels of normalization can be used?
- How to choose a good reference gene?

10.15-10.30
Coffee Break

10.30-11.45
Experiment comparing different quantification strategies

- relative and standard curve quantification
- different efficiency calculations/assumptions

11.45-12.45
Lunch

12.45-13.30
Optimization of qPCR protocols

- What parameters can/should be optimized?
- An optimization strategy

13.30-15.00
Quantification calculation examples

- what effect will efficiency have on quantification
- quantification methods, and equations

15.00-15.15
Coffee Break

15.15-16.45
Analysis of experimental data

- differences in quantifications strategies
- effect of efficiency estimations on results
- calculations of relative abundance of genes
- pros and cons of different methods

16.45-17.00 **Discussion and Q&A**

17.00 End of qPCR workshop day 2

Fri. 9th September

Day 3 - Advanced qPCR: Sample Preparation and reverse transcription

09.00-10.00
Principle of RT and different RT priming strategies

- Pros and cons of different methods

10.00-10.45
Principle of RNA and DNA extraction

- How it works
- Available methods and products suitable for qPCR
- Practical considerations

10.45-11.00
Coffee Break

11.00-11.45
Reverse transcription experiment using different priming methods

- Oligo(dt)
- Random Hexamers
- Gene specific primers

11.45-12.45 Lunch

12.45-13.30
qPCR experiment evaluating RT using the generated cDNA

- Is there a best RT priming method?

13.40-14.30
Quality Control in qPCR using Kinetic Outlier Detection

- How to detect samples with significant inhibition

14.30-14.45
Coffee Break

14.45-15.30
SNP detection. Multiplexing possibilities and problems

- qPCR for SNP/mutation detection. What alternatives are there?
- Multiplex optimization

15.30-16.15
Analysis of experimental data

- Which priming method for RT is best?
- How should experiments be planned to take RT priming into consideration?

16.15-16.30
Probes and Dyes

- What dyes/quenchers are typically used in qPCR
- How to measure the maximum fluorescence available in a dual-labelled probe

16.30-16.45 **Discussion and Q&A**

16.45 End of qPCR workshop day 3

qPCR Matrix Workshop

qPCR Matrix Workshop from 7th – 9th is a new type of interactive qPCR workshop in practical rooms **P1, P3, and P4**

- Researchers and qPCR companies are addressed to present their technologies (cycler, chemistry, kits or software applications) by their own product application specialists.
- Each student can visit **6 different session types** in **6 time frames.**
- After finishing a session the students move to the next bench and session type.
- In each time session and frame **4-6 students** can be educated by **1-2 session instructors.**
- Each session will be held on two lab-benches, one for sample preparation and one for the qPCR applications.
- Each workshop session will last for **3-4 hours**.
- Maximal three sessions will be presented at one day.
- **The students can choose and book the sessions at the meeting registration desk on Monday afternoon 5th Sept.**

Researchers and qPCR companies will be responsible for their own equipment: all hardware, software, kits and disposables. Workshop will take place in practical rooms P1, P3, and P4 with different number of workshop benches, chairs, electricity, W-LAN, fridges, freezers, crushed ice, water, etc.….

Matrix Workshop time table:

Wednesday afternoon 7th Sept.

Opening of the Workshop and introductory talk by the WS organizers	**13:00 – 14:00**
coffee break	14:00 – 14:30
time frame 1:	**14:30 – 18:30**

Thursday 8th Sept.

time frame 2:	**08:30 – 11:30**
Lunch in the cafeteria	11:30 – 12:30
time frame 3:	**12:30 – 15:30**
variable coffee break	
time frame 4:	**15:30 – 18:30**

Friday 9th Sept.

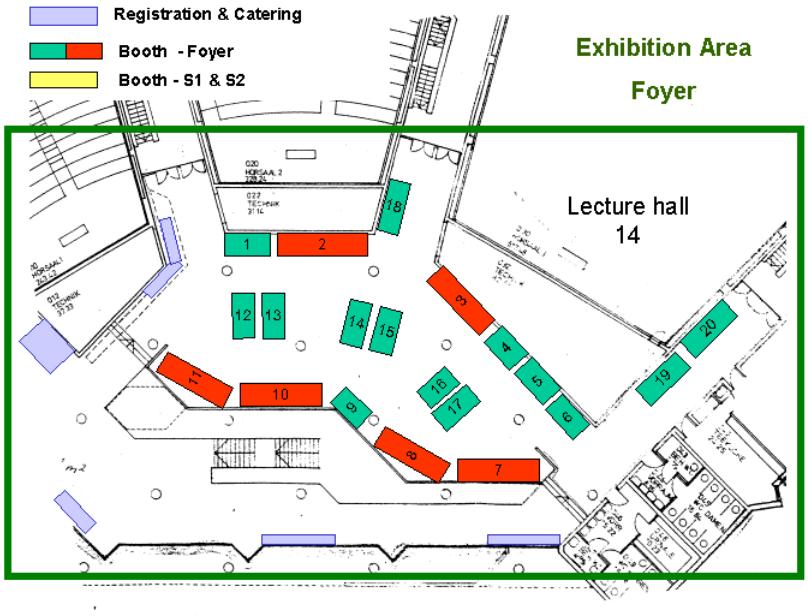
time frame 5:	**08:30 – 11:30**
Lunch in the cafeteria	11:30 – 12:30
time frame 6:	**12:30 – 15:30**
Closing of the workshop	15:30 – 16:00

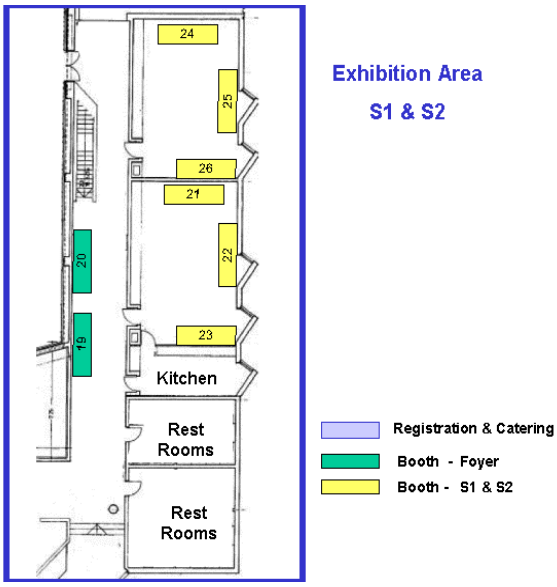
Announced qPCR Workshop sessions:

Room	Session Instructor, Institution Company	Session title and description
Application P1	**Andrés Jarrin** Application specialist, Eppendorf AG	Reaction setup and quantitative PCR analysis with the **Eppendorf epMotion liquid handling workstation** and **Mastercycler realplex**.
Application P1	**Christine Munz & Dierk Evers** Roche Applied Science, Mannheim, Germany	Accurate Gene Expression Analysis with High Flexibility: **LightCycler® 480 System and Universal ProbeLibrary.**
Application P1	**Martin Schlumpberger** **Ina Scheuerpflug** QIAGEN GmbH, R&D, Hilden, Germany	**Easy Isolation of total RNA** from formalin-fixed, paraffin-embedded tissue sections and SYBR Green qPCR with new, ready-to-use QuantiTect Primer Assays. Time frame: 1 and 3 and 5
	Dirk Loeffert & Annette Tietze QIAGEN GmbH, R&D, Hilden, Germany	Fast and efficient: **Synthesis of cDNA without RNA isolation** and reliable real-time multiplex PCR. Time frame: 2 and 4 and 6
Application P1	**Thomas Kaiser & Rudolf Walser** LTF-Labortechnik, Germany, Corbett Research, Australia	Primer & probe matrix optimization and PCR setup using the **CAS-1200 and high speed runs on the Rotor-Gene**.
Application P1	**Steffen Müller** Stratagene, Field Application Scientist	FastPCR - Speed up results with **FullVelocity**.
Application P1	**Marcus Neusser** Gene Expression Devision, Bio-Rad Laboratories	**Fast PCR Applications: Sense and Nonsense** Success with SYBR - How to Design and Implement High-Quality SYBR Green I Reactions.
Application P4	**Kyle Hooper & Alyssa TenHarmsel** Promega Corporation, Woods Hollow Road, Madison, WI, USA	Simplify **multiplexed qPCR analysis** a new approach with the **Plexor** Real-Time PCR System.
Application P4	**Astrid Potratz** Support Specialist, Applied Biosystems, Germany	From Tissue to a **Gene Expression Profile** in less than 5 hours. Using the **Applied Biosystems 7900 Real-time PCR System** in combination with the **ABI Prism 6100 Nucleic Acid PrepStation**.
Application P4	**Andreas Eckelt** Cepheid SA, Odenthal, Germany	**Efficient and fast transition of a gel-based to a real time PCR reaction.** Content: a standard PCR reaction will be converted to a real-time PCR assay. Temperature profile, buffer conditions and primer concentration will be adopted.
Application P4	**Mark Andersen** R&D Scientist, Invitrogen, USA	Analyze your SNP genotype and buccal cell expression profile with **CellsDirect(TM) and LUX(TM) detection technology**.
BioInformatics P3	**Michael W. Pfaffl and colleagues** Physiology Weihenstephan, TUM, Germany	Application of various new REST software versions: Time frame: 1 and 5 **REST-384** (REST high throughput) **REST-MCS** (Multiple Condition Solver) **REST-RG** (REST for Rotor-Gene) **REST-2005** (REST stand alone application)
	Jo Vandesompele & Jan Hellemans Center for Medical Genetics Ghent University Hospital, Belgium	Application of normalization and calculation software: **geNorm** **qBASE** Time frame: 2 and 3
	Mikael Kubista and collegues MultiD Analyses AB, Lotsgatan, Göteborg, Sweden	**GenEx** for real-time PCR data analysis and sample classification. Time frame: 4 and 6

Industrial Exhibition

An industrial exhibition will be held during the qPCR Symposium from **5 – 7th September** in the foyer of the central lecture hall complex (green frame) and in two seminar rooms S1 and S2 (blue frame).





Nr 1: Metabion	Nr 2: Roche Applied Science	Nr 3: Eppendorf	Nr 4: Eurogentec
Nr 5: Bioline	Nr 6: Operon Biotechnologies	Nr 7: Applied Biosystems	Nr 8: Stratagene
Nr 9: MWG Biotech AG	Nr 10: Bio-Rad	Nr 11: LTF Labortechnik / Corbett Research	
Nr 12: Abgene	Nr 13: Cepheid	Nr 14: Qiagen	Nr 15: New England Biolabs
Nr 16: Tib Molbiol	Nr 17: Invitrogen	Nr 18: Promega	Nr 19: MP Biomedicals
Nr 20: Biosearch Technologies	Nr 21: Agilent Technologies	Nr 22: Chimera Biotec	Nr 23: RoboScreen
Nr 24: Fujifilm	Nr 25: Nugen Inc.	Nr. 26: Gene Express Inc.	

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ISBN 3000166874

Proceedings

qPCR 2005
2nd International qPCR Symposium & Industrial Exhibition
TATAA Application Workshop & qPCR Matrix Workshop
The whole story of quantitative PCR – from Tissue Preparation to BioInformatics