3rd – 6th March 2004 in Freising-Weihenstephan, Germany

1st International qPCR Symposium & Application Workshop

Symposium Proceedings

1st International qPCR Symposium & Application Workshop

Editor: Michael W. Pfaffl

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Invitation to the 1st International qPCR Symposium & Application Workshop

Dear researchers and colleagues,

On behalf of the Organisation Committee and the Scientific Board it is great pleasure to invite you to the **1st International qPCR Symposium & Application Workshop** to be held March 3-4, 2004, at the Center of Life Science in Freising Weihenstephan, Technische Universität München Germany. (http://www.wzw.tum.de/wzw/english/index.html).

The polymerase chain reaction (PCR) is the most sensitive technique to detect low abundance mRNA and minute amounts of DNA typically present in tissue samples. Real-time reverse transcription PCR (qPCR or kinetic RT-PCR) is rapidly becoming the method of choice for quantitative gene expression analysis and molecular diagnostics owing to its high sensitivity, excellent reproducibility, and wide dynamic quantification range. However, this ultra high sensitivity makes qPCR sensitive to experimental error, variations in experimental protocols and presence of contaminants in complex biological samples. Successful applications of qPCR require understanding of the practical problems associated with sample handling, including careful experimental design, assay optimization, appropriate validation and proper data treatment.

This **1st International qPCR Symposium** 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 and associated **Application Workshop** offers an overview of the present knowledge and future developments in qPCR technology and its wide applications.

The **Application Workshop** offers hands-on training by leading experts from TATAA Biocenter (www.tataa.com) in qPCR. Major probe technologies will be demonstrated, leading and emerging instrument platforms will be presented, and advanced primer and probe design programs will be available. Hands-on training will be provided on quantitative gene expression, including design and optimiszation of both RT and qPCR protocols.

Michael W. Pfaffl
(Chairman Symposium)

Mikael Kubista
(Chairman Application Workshop)

Industrial Exhibition

More than **32 companies** will participate in the **Industrial Exhibition** along with the Symposium.
(real-time PCR Cyclers, Kit Producers, Nucleic Acid Purification Systems, Enzyme Producers, Plasticware Suppliers, Software Developers, etc.)

Company details under http://www.wzw.tum.de/gene-quantification/qpcr2004/companies.html

Press release – English

1st International qPCR Symposium & Application Workshop

Transcriptomics, Clinical Diagnostics & Gene Quantification

The 1st International qPCR Symposium & Application Workshop is organized jointly by the Technical University of Munich and the TATAA Biocenter, Sweden. http://www.wzw.tum.de/gene-quantification/qpcr2004/

The Physiology Weihenstephan at the Technical University of Munich and the TATAA Biocenter in Sweden, with support from leading biotechnology companies, have taken the initiative to gather for the first time World's leading researchers in the qPCR field. During 4 days in Freising-Weihenstephan more than 450 scientists from 41 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.

Quantitative real-time polymerase chain reaction, qPCR, is an improved technology based on PCR that was awarded 1993 years Nobel price in Chemistry. With 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 only one molecule. The technique has revolutionized molecular diagnostics. Hospital laboratory tests that used to take weeks, sometime months to perform, and required the handling of hazardous chemicals, can today be made in hours in fully automated systems. Conference presentations will show that in near future, using improved instrumentation, the qPCR test will take only 15 minutes from sampling, and the test results will be delivered to the patient while waiting. Doctors will be able to perform tests during surgery and decide treatment based on test results. In biomedical research and drug development gene expression measurements with qPCR opens completely new possibilities. Presentations at the conference will show how expression of key genes can be measured in individual cells. This makes it possible to study development processes, including the differentiation of stem cells into specialized tissue. Global expression of genes reflects the health state of an individual, which makes it possible to monitor how patients respond to drugs. This will lead to individual treatments of disease and ultimately to individualized medicine.

Identification and quantification of pathogens in plant, animal and human deseases – gene silencing, pharmacogenomics, nutrigenomics - there is almost no field in life sciences not open to many qPCR applications for nucleic acid analysis. Further developments of qPCR technology focus on miniaturisation, higher throughput, cost efficacy and validity. Combination of qPCR with mass spectrometry allows the rapid detection of point mutations; combination with reverse transcription enables determination of RNA and widely opens the window for *"Transcriptomics"* – the first step of gene expression and functional genomics. Some respective highlights of the conference: expression of anti aging genes becomes measurable - sensitivity and response to malaria shows individual differences. qPCR has allowed to illuminate the gap between encoding gene and final gene product.

About the Physiology Weihenstephan at the Center of Life and Food Sciences at Technical University of Munich: The Physiology Weihenstephan chaired by Professor 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 and growth. Both traditional endocrinology and paracrine regulations are studied. Dr. Michael W. Pfaffl is developing qPCR for quantitative gene expression analysis, optimizes and validates the qPCR reaction (http://www.wzw.tum.de/gene-quantification/).

About TATAA Biocenter: TATAA Biocenter (http://www.tataa.com) is the leading qPCR service provider in Europe. It has contributed to the development of qPCR by several inventions, including the LightUp probes that are used in the RESSQ assays for human infectious disease testing by LightUp Technologies AB, the qPCR lymphoma test developed by CanAg diagnostics AB, and the BEBO family of dyes for non-specific labeling of qPCR products. The center is associated with Chalmers University of Technology and the University of Göteborg, in Sweden.

For more information about the 1st International qPCR Symposium & Application Workshop, see: http://www.wzw.tum.de/gene-quantification/qpcr2004/ or contact Dr. Michael W. Pfaffl (pfaffl@wzw.tum.de), Professor Heinrich H. D. Meyer (physio@wzw.tum.de), or Professor Mikael Kubista (mikael.kubista@tataa.com).

Pressemitteilung - Deutsch

1st International qPCR Symposium & Application Workshop

Transkriptomics, Klinische Diagnostik & Gen Quantifizierung

Das „1st International qPCR Symposium & Application Workshop" wird vom Lehrstuhl für Physiologie, Technische Universität München und dem TATAA Biocenter aus Schweden als gemeinsame Veranstaltung durchgeführt und von der TUM-Tech GmbH organisatorisch unterstützt. http://www.wzw.tum.de/gene-quantification/qpcr2004/

Der Lehrstuhl für Physiologie an der Technischen Universität München und das TATAA Biocenter in Schweden haben mit Unterstützung durch führende Biotechnologieunternehmen die Initiative ergriffen weltweit agierende Wissenschaftler auf dem Feld der quantitativen PCR zusammenzubringen. Während der 4 Konferenztage in Freising-Weihenstephan werden mehr als 400 Wissenschaftler aus 41 Nationen ihre Ideen austauschen, Erfahrungen teilen und die faszinierende Zukunft dieser innovativen Technik diskutieren.

Die quantitative Polymerase-Ketten-Reaktion (qPCR) ist eine weiterentwickelte Technologie basierend auf der PCR, die 1993 mit dem Nobelpreis für Chemie ausgezeichnet wurde. Mit Hilfe der qPCR können Nukleinsäuren in komplexen Proben mit höchster Präzision und hervorragender Spezifität bestimmt werden. Diese außergewöhnliche Sensitivität erlaubt die Erfassung von nur einem einzigen DNA Molekül, sodass diese Technik die Molekulare Diagnostik grundlegend verändert hat. Tests in klinischen Laboratorien, die früher Wochen sogar Monate benötigt haben und die Handhabung gefährlicher Chemikalien erforderten, können heutzutage in einer Stunde in vollständig automatisierten Systemen durchgeführt werden. Einzelne Präsentationen der Konferenz zeigen den Weg wie in naher Zukunft mit weiterentwickelter Instrumentalisierung qPCR Tests in 15 Minuten durchgeführt werden können von der Probennahme bis zum Ergebnis. Die Befunde liegen schon vor, während der Patient noch wartet. Ärzte werden die Möglichkeit haben, Tests während der Operation durchzuführen und auf der Basis des Testresultates über die Behandlung zu entscheiden. Die Messung der Genexpression mittels qRT-PCR

eröffnet völlig neue Möglichkeiten für die Biomedizin und Medikamentenentwicklung. Die Präsentationen auf der Konferenz zeigen wie die mRNA Expression von Schlüsselgenen in einzelnen Zellen erfasst werden können. Dies ermöglicht, entwicklungsbiologische Prozesse im Detail zu untersuchen und die Differenzierung von Stammzellen zu spezialisiertem Gewebe zu verstehen. Die Reaktion von Patienten auf Medikamente lassen sich über Markergene erfassen, die für den Gesundheitsstatus charakteristisch exprimiert werden. Dies eröffnet völlig neue Horizonte bei der individuellen Behandlung von Erkrankungen, der Individualmedizin.

Identifizierung und Quantifizierung von Pathogenen bei Erkrankungen von Pflanze, Tier und Mensch - Gene Silencing, PharmakoGenomics, NutriGenomics – es gibt fast keinen Arbeitsbereicht in den Lebenswissenschaften für den die qPCR nicht neue Möglichkeiten der Nukleinsäureanalytik eröffnet hat. Die weiteren Entwicklungen der qPCR konzentrieren sich auf die Miniaturisierung, hohen Probendurchsatz, Genauigkeit, Optimierung der Kosten-Nutzen-Effizienz sowie deren Zuverlässigkeit. Die Kombination von qPCR und Massenspektrometrie erlaubt die schnelle Erkennung von Punktmutationen.

QPCR in Kombination mit der reversen Transkription, ermöglichen die exakte Bestimmung von mRNA und führt zu den „Transkriptomics“ – den ersten Schritt der Genexpression und der funktionalen Genomanalyse. Einige entsprechende Highlights der Konferenz: Die Expression von Anti-Alterungsgenen wird messbar – die Sensitivität und die Reaktion auf Malaria zeigt individuelle Unterschiede. Die Kenntnislücke zwischen dem kodierenden Gen und dem finalen Genprodukt kann nun mit Hilfe der qPCR angegangen werden.

Zum Lehrstuhl für Physiologie am Wissenschaftszentrum für Ernährung, Landnutzung und Umwelt Weihenstephan an der Technischen Universität München: Der Lehrstuhl für Physiologie unter der Leitung von Herrn Prof. Heinrich H. D. Meyer ist eine führende Einrichtung im Bereich der Molekularen Physiologie der Säugetiere. Schlüsseltechniken der Molekularbiologie und Biochemie werden eingesetzt für die Erforschung von Grundlagen und anwendungsorientierten Problemen auf den Gebieten der parakrinen und endokrinen Regulation von Fortpflanzung, Laktation und Wachstum. Die Arbeitsgruppe von Dr. Michael W. Pfaffl optimiert und validiert die qRT-PCR und entwickelt neue Strategien auf dem Gebiet der quantitativen Genexpressionsanalytik (http://www.wzw.tum.de/gene-quantification/).

Zum TATAA Biocenter, Schweden: Das TATAA Biocenter (http://www.tataa.com) unter der Leitung von Herrn Prof. Mikael Kubista ist einer der führenden qPCR Dienstleister in Europa. Das Forschungszentrum ist an die Chalmers University of Technology sowie die Universität von Göteburg angegliedert. Die folgenden qPCR Innovationen und Erfindungen wurden vom TATAA Biocenter entwickelt und vorangetrieben: die LightUp Probes, der RESSQ Assays für die humane Infektionsdiagnoastik durch LightUp Technologies AB, der qPCR Lymphomatest entwickelt durch CanAg Diagnostics AB, und die BEBO Farbstoffe für die unspezifische Markierung von qPCR Produkten.

Weiter Informationen über die Tagung: http://www.wzw.tum.de/gene-quantification/qpcr2004/ oder direkt bei Dr. Michael W. Pfaffl (pfaffl@wzw.tum.de), Professor Heinrich H. D. Meyer (physio@wzw.tum.de), und Professor Mikael Kubista (mikael.kubista@tataa.com).
Kongressorganisation: TUM-Tech GmbH, Dr. Ulrich Wild (ulrich.wild@tumtech.de)

Download Symposium Contributions

More than **65 internationally reknown speakers** and over **135 poster contributions** in **8 specialised poster sessions** will participat in a lively and exciting programme enabling the valuable exchange of information in the qPCR field.

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465 participants and around 200 abstracts from every place on earth were send to us.

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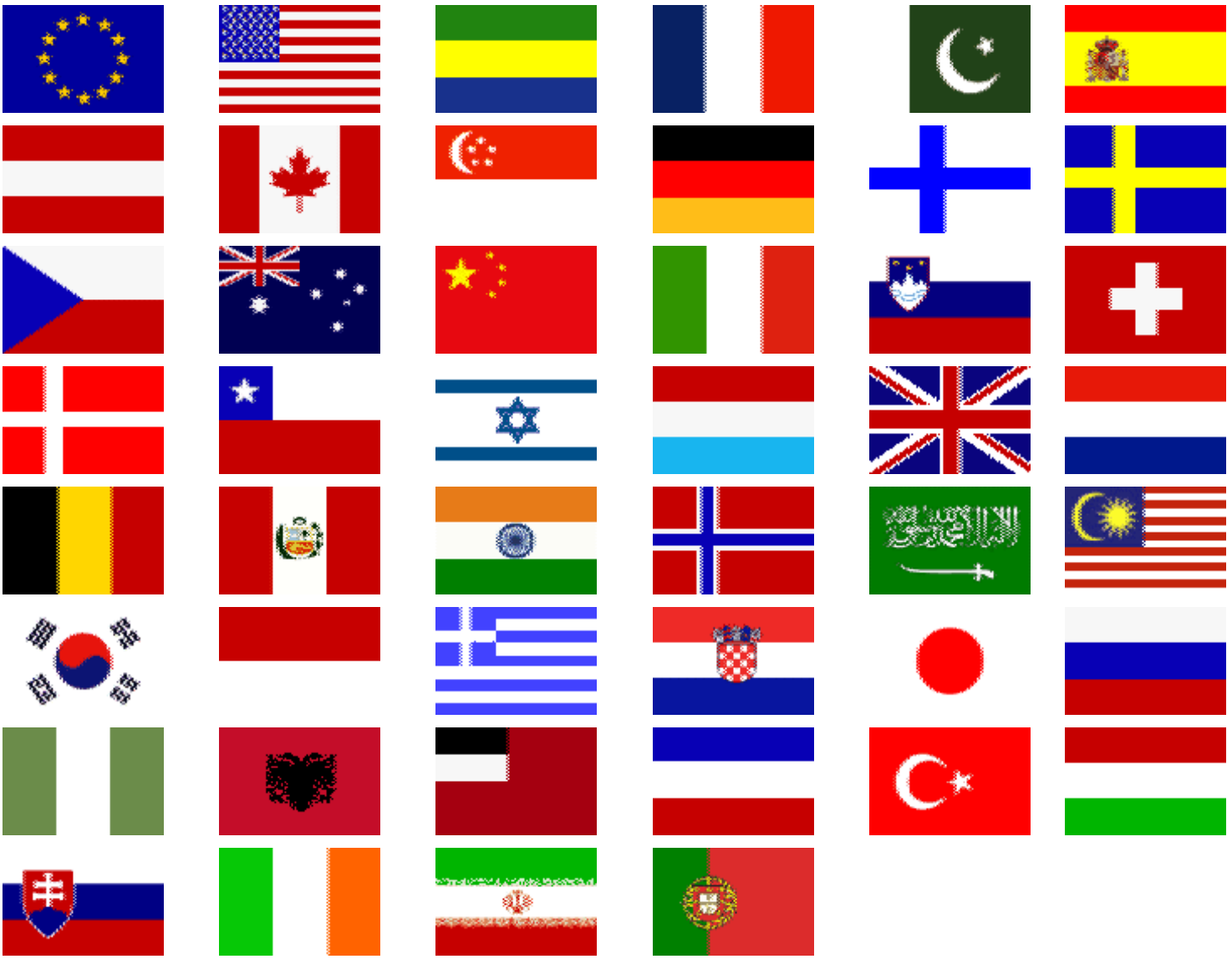

Agenda

Wednesday 3 March 2004

Welcome & Opening of the Symposium
Lecture hall HS 14

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

10:00 – 11:30 Arrival & Registration

11:30 – 12:30 **Get-together lunch in the student cafeteria**

12:30 "Welcome & Opening of the Symposium."
Michael W. Pfaffl & Mikael Kubista, Scientific coordination of the Symposium & Workshop

12:45 "Welcome at the Center of Food & Life Science in Freising Weihenstephan."
Bertold Hock, Dean of the Center of Food & Life Science-Weihenstephan, Freising, Germany

13:00 "qPCR and Transcriptomics: Creation of a new tool to understand life."
Heinrich H. D. Meyer, Physiology - Weihenstephan, Freising, Germany

13:45 "Pitfalls in the quantification of RNA using real-time RT-PCR."
Stephen Bustin, Reader in Molecular Medicine, School of Medicine, London

14:30 – 15:00 **Coffee break**

"qPCR and Transcriptomics: Creation of a new tool to understand life."

Heinrich H. D. Meyer (physio@wzw.tum.de)

Physiology – Weihenstephan, Freising, Center of Food & Life Science – Weihenstephan, Germany

The earlier methods for DNA and RNA quantification – southern and northern blots – had many limitations and the problems associated with these techniques like the need of radioisotopes where the driving force for the development of qPCR. This started about 15 years ago with internal and external standardisation methods. Optimal absolute quantification was possible with competitive qPCR using modified internal standards but identical primers. Still separation via electrophoresis or HPLC was required. The development of real-time PCR about eight years ago started the breakthrough for qPCR. This method nowadays enables measuring of new analytes very rapidly and without big efforts. DNA or gene quantification is important in many fields like clinical diagnostics, food hygiene or biotechnology. Even more, expression profiling and transcriptomics became widely opened by real-time qPCR – this technology allows determination limits down to a few RNA molecules in single cells and gives ways to understand many regulatory mechanisms. qPCR is used in endocrinology, for paracrine regulation, for pathophysiology and for many other applications in microbiology, plant, animal and human sciences – even anti aging genes can be investigated now. About forty years ago radio immuno assays (RIA) provided the possibility to measure trace amounts of proteo and steroid hormones. qPCR nowadays fills the gap of trace analysis on the transcription level. Gene silencing, siRNA and other modified nucleic acids are a further fascinating field investigated by qPCR.

Numerous approaches for internal validation of qPCR have been published in the meantime and in addition qPCR can used for the validation of array technologies that provide much less sensitivity and precision. Present technological developments mainly focus on new detection methods like duplex or multiplex PCR or new dyes, on miniaturisation or high throughput and on new combinations. PCR/MS allows the precise identification of the PCR products and much better differentiation like earlier electrophoresis methods.
In conclusion real time qPCR has become a superior cost effective technology that allows nucleic acid analyses with great sensitivity and validity in the whole field of life sciences.

"Pitfalls in the quantification of RNA using real-time RT-PCR."

Stephen Bustin (s.a.bustin@qmul.ac.uk)

Reader in Molecular Medicine, School of Medicine, London

PCR-based assays can target either DNA (the genome) or RNA (the transcriptome). Targeting the genome generates robust data that are informative and, most importantly, generally applicable. This is because the information contained within the genome is context-independent, *i.e.* generally every normal cell contains the same DNA sequence, the same mutations and polymorphisms. The transcriptome, on the other hand, is context-dependent *i.e.* the mRNA complement and level varies with physiology, pathology or development.

This makes the information contained within the transcriptome intrinsically flexible and variable. The heterogeneity of *in vivo* biopsies, together with the inadvertent changes to expression profiles caused by the handling of the samples prior to RNA preparation also can interfere with results, especially quantitative expression profiling data.

If this variability is combined with the technical limitations inherent in any RT-PCR assay, it becomes clear that it can be difficult to achieve not just a technically accurate but a biologically accurate result. Template quality, operator variability, the RT-step itself and subjectivity in data analysis and reporting are just a few technical aspects that make real-time RT-PCR appear to be a fragile assay. can make accurate data interpretation difficult.

There can be little doubt that in the future transcriptome-based analyses will become a routine technique. However, for the time being they remain research tools and it is important to recognise the considerable pitfalls associated with transcriptome analysis, with the successful application of reverse-transcription (RT)-PCR depending on careful experimental design, application and validation.

Session: Pre-Analytical Steps
Chairs: M. Kubista & D. Grove
Lecture hall HS 14

15:00 Session introduction by Mikael Kubista & Deborah Grove

15:10 "Quantitative gene expression analysis by Real-time PCR - How to optimize the reverse transcription and real-time PCR reactions."
Anders Stahlberg, Mikael Kubista, Chalmers University, Sweden

15:40 "Overcoming bias in quantitative RT-PCR: Different methods of reverse transcription influence sensitivity and accuracy of gene expression patterns."
Ginzinger DG[1], Yu M[1], Schuster D[2] & A Rashtchian[2]; University of California San Francisco, Comprehensive Cancer Center, Genome analysis core facility, San Francisco, CA, USA; [2] Quanta BioSciences, Inc. Gaithersburg, MD 20877, USA

16:00 "RNA Integrity Number (RIN) –Standardization of RNA Integrity Measurements."
Odilo Mueller[1], Andreas Schroeder[1], Samar Lightfoot[2], Ruediger Salowsky[1], Susanne Stocker[1], Thomas Ragg[3]; 1 Agilent Technologies, Waldbronn, Germany; 2 Agilent Technologies, Palo Alto, USA; 3 Quantiom Bioinformatics, Weingarten, Germany

16:20 "Gene expression quantitated in PAXgene™ frozen stored blood as compared to freshly Immuno Magnetic Separated (IMS) blood cells."
Ovstebo R, Haug KBF, Kierulf P.; The Research and Developement Group, Department of Clinical Chemistry, Ulleval University Hospital, Oslo, Norway

16:40 "Modified silica-magnetite composite as a universal matrix for nucleic acids isolation."
Zhao X, Huang Z, Luan G; Biovision Biotech, Inc, China

17:00 – 18:00 **Poster session**

18:00 – 22:00 ***Get-together with the Companies***
Bavarian Dinner Buffet in the Aula

"Quantitative gene expression analysis by Real-time PCR - How to optimize the reverse transcription and real-time PCR reactions."

Anders Stahlberg, Mikael Kubista, Chalmers University, Sweden
(anders.stalberg@molbiotech.chalmers.se)

Reverse transcription quantitative real-time PCR, or RT-QPCR for short, is the method of choice for quantitative studies of gene expression. The method has been an important tool in research for some years, and is now starting to replace conventional methods in routine diagnostics. Real-time PCR is characterized by wide dynamic range of quantification, high sensitivity, and high precision. However, gene expression analysis

requires that mRNA is first reverse transcribed to cDNA. This reaction can introduce errors that bias the result due to effects of mRNA secondary and tertiary structures, variation in priming efficiency, and properties of the reverse transcriptase. We have studied the properties of the reverse transcription reaction for quantitative gene expression measurements. In this talk these results will be presented and summarized in terms of rules how to optimize RT-QPCR measurements. A number of applications will also be presented, including diagnosing non-Hodgkin lymphomas by measuring the relative expression of two tumor markers, determining the number of mRNA copies in individual cells, and monitoring transcriptional response over time after external stimuli of a yeast population.

"Overcoming bias in quantitative RT-PCR: Different methods of reverse transcription influence sensitivity and accuracy of gene expression patterns."

Ginzinger DG[1], Yu M[1], Schuster D[2] & A Rashtchian[2]; (dginzing@cc.ucsf.edu)

[1] University of California San Francisco, Comprehensive Cancer Center, Genome analysis core facility, San Francisco, CA 94143, USA
[2] Quanta BioSciences, Inc. Gaithersburg, MD 20877, USA

Real-time quantitative RT-PCR has become a mainstream technique for the measurement of gene expression levels, yet very little effort has been devoted to the reverse transcription component of this process. The lack of attention to this critical portion of the process can be at the peril of robust and reliable data and introduce potential bias which may be attributed to differential gene expression.
Here we present a comprehensive analysis of five different reverse transcription methods on different breast cancer cell lines and subsequent expression analysis of three genes. The reverse transcriptase reactions were performed according to three different commercially available kits (kits A, B and C), our in-house Core standard protocol and iScript cDNA synthesis kit. Each protocol was tested on two to four different breast cancer cell lines, including; MCF7, SKBR3, T47D and MDA360, and subsequent gene expression analysis was performed with TaqMan assays for GAPDH, GUS and ZNF217 (a putative oncogene upregulated in breast cancer). Measures of valid RT reactions are, overall raw Ct values for all genes for each cDNA, RT linearity (analogous to PCR efficiency measurements) as well as consistent relative expression values over a wide range of RNA input amounts (2ug to 20 ng). For equal amounts of RNA input into the RT reaction and subsequent real-time PCR reaction the iScript method had the over all lowest Cts for all genes in all RNAs followed very closely by the Core standard protocol and the kit "B". The other two methods from kits A and C varied dramatically and were on average more than 1 Ct higher and up to 3 Cts (or 10 fold) in some cases. The critical factor of RT linearity, as measured from the slope of Ct values versus log input cDNA concentration (similar to PCR efficiency calculations), was best for the widest range of RNA input amounts for the iScript method (ranging from ~700 to 20 ng and in one case, MCF7, up to 2ug) followed closely by the Core standard protocol (~700 ng to 44 ng). However, company B only had a linear range from about 375 to 44 ng and remarkably the other two protocols rarely gave any linear results whatsoever. Finally, the relative gene expression of ZNF217 (relative to GAPDH) in MCF7 cells using the Core's standard protocol is 11%± 3% for all RNA input levels (1.4ug to 22 ng and is consistent with numerous other previously published data). Using the iScript method, ZNF217 was expressed at 10%± 2% for all RNA input levels. By contrast ZNF217 is 6.2%± 2%, 6.3%± 2% and 15% ± 5% the level of GAPDH for kits "A", "B" and "C" respectively.
In conclusion, these data demonstrate a wide range of apparent expression levels for the same genes in the same cell lines depending on the RT protocol is used. Furthermore, the linearity for the range of RNA input amounts can vary considerably. Together, these data have convinced us that the most robust, reliable and sensitive method for generating cDNA is with the iScript method. Although similar to our current method with respect to sensitivity and relative expression levels, iScript provides a wider linear range and is much easier to use. Therefore, our core and most of the investigators we support have switched to this RT protocol and will benefit from the standardization of RT protocols thereby making data much more comparable between labs.

"RNA Integrity Number (RIN) –Standardization of RNA Integrity Measurements."

Odilo Mueller[1], Andreas Schroeder[1], Samar Lightfoot[2], Ruediger Salowsky[1], Susanne Stocker[1], Thomas Ragg[3]; (odilo_mueller@agilent.com)

1 Agilent Technologies, Waldbronn, Germany
2 Agilent Technologies, Palo Alto, USA
3 Quantiom Bioinformatics, Weingarten, Germany

RNA quality assessment has been identified as one of the most critical elements in order to obtain meaningful gene expression data via microarray or real-time PCR experiments. Current advances in microfluidics have improved RNA quality measurements tremendously allowing a more detailed look at RNA degradation patterns by providing electrophoretic traces of RNA samples. However, the interpretation of such electropherograms still requires a certain level of experience and can vary from one researcher to the next.
A so-called "RNA integrity number" (RIN) algorithm is introduced that assigns 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. It was found that the RIN is more reliable than the ribosomal ratio, when assessing the integrity of RNA samples. The RIN is shown to be largely independent of RNA concentration, instrument (Agilent 2100 bioanalyzer), and most importantly, the origin of the RNA sample. Using the RIN, researchers can work towards standardization of the important RNA integrity measurement ensuring reproducibility and reliability of gene expression experiments.

"Gene expression quantitated in PAXgene™ frozen stored blood as compared to freshly Immuno Magnetic Separated (IMS) blood cells."

Ovstebo R, Haug KBF, Kierulf P
reidun.ovstebo@ioks.uio.no

The Research and Developement Group, Department of Clinical Chemistry, Ullevål University Hospital, Oslo, Norway.

Introduction: Changes in gene expression patterns in circulating white blood cells may be observed during various disease states such as infections, myocardial infarction, traumas and autoimmune diseases. Quantitative estimates of specific mRNAs may provide new insights in these disease issues. The convenience of just drawing a blood sample makes it interesting to get hold of samples that can be used to establish biobanks for gene expression studies in epidemiological populations. This requires special treatment of the blood samples to obtain high quality. Aim: To evaluate the use of PAXgene™ vacuum tubes for robotized isolation of high quality mRNA from whole blood stored at 4 °C and -70 °C. Furthermore to compare the gene expression patterns obtained from WBC stored in PAXgene™ or isolated with IMS.
Material and Methods:Heparinized whole blood was stimulated with LPS (0,100, and 1000 pg/ml) for 4 hours. The blood was either stored in PAXgene™ tubes (Qiagen) at 4°C or -70°C or immediately subjected to isolation of WBC by IMS (CD45 and CD 15 beads) (Dynal Biotech ASA, Norway), lysed and stored at -70°C. PAXgene™ and IMS lysates were subsequently subjected to robotized RNA isolation (QIAsoft M, BioRobot M48, Qiagen)and qRT-PCR amplified(1) to obtain quantitative estimates of constitutive and LPS-inducible gene expression.
Results:

- Frozen blood cells stored in PAXgene™ appear to give the same levels of gene expression as blood stored in PAXgene™ at 4 °C when evaluated by qRT-PCR.
- Blood cells stored in PAXgene™ appear to give fairly the same levels of gene expression as IMS isolated WBC when evaluated by qRT-PCR.

Conclusions:
The integrity and quality of RNA isolated from frozen PAXgene™ tubes appears satisfactory. Furthermore, gene expression from frozen and freshly isolated blood cells appears to be similar comparable. PAXgene™ tubes may thus be usable for storage of blood cells for biobanking and subsequent RNA studies..

1. Ovstebo R, Haug KB, Lande K, Kierulf P. PCR-based calibration curves for studies of quantitative gene expression in human monocytes: development and evaluation. Clin Chem 2003;49:425-32.

"Modified silica-magnetite composite as a universal matrix for nucleic acids isolation."

Zhao X, Huang Z, Luan G (neil_x_zhao@yahoo.com)

Biovision Biotech, Inc, China

Silica-magnetite composite were prepared and modified as a universal adsorbent for the isolation of DNA and RNA of different types from various sources. A novel method was described for the simple preparation of borate-silica–magnetite composite, which afford higher yields of DNA than when using silica–magnetite alone, or a commercially available kit. This composite could be used for PCR product purification, gel-electrophoresis separated DNA fragments recovery, human genomic DNA isolation from either cell or whole blood, plant genomic DNA isolation from various sources, and virus DNA/RNA purification from sera. Furthermore, with an additional surface modification with hydrophobic groups and streptavidin conjugation, these composites can be used for mRNA or species-specific RNA isolation from different sources. Nucleic acids isolated using the borate-silica–magnetite was suitable for use in further applications such as PCR and enzyme digestion. The precision of isolation was evaluated with real-time PCR or real-time RT-PCR, the results showed that the developed matrix was suitable for various types of nucleic acids with high reproducibility. Also, the developed matrix could be adapted to automatic nucleic acids purification instruments for high throughput nucleic acids extraction.

Thursday 4 March 2004

Session: qPCR Application in Clinical Diagnostics
Chairs: S. Bustin & M. Pazzagli
Lecture hall HS 14

08:30 Session introduction by S. Bustin & M. Pazzagli

08:40 "Real-time PCR is the most sensitive technique for biomolecular detection. Possibilities and Limitations in Research and in Clinical Diagnostics."
Mikael Kubista, Chalmers University of Technology and the TATAA Biocenter, Göteborg, Sweden

09:10 "Laser capture microdissection and real-time PCR in Human Cancer."
Pamela Pinzani, Prof. Mario Pazzagli, Clinical Biochemistry Unit, Dep. of Clinical Physiopathology Florence, Italy

09:40 "Microarray data of pooled samples compared to real-time-PCR of individual samples in African Children with Malaria."
Yvonne Kalmbach (1), Angelica B. W. Boldt (1), Martin P. Grobusch (1,2), Cristina Tena-Tomás (1), Arnaud Dzeing (4), Maryvonne Kombila (4), Michael Bonin (3), Olaf Riess (3), Peter G. Kremsner (1,2), Jürgen F. J. Kun (1). Institute for Tropical Medicine, Dept. of Parasitology, Wilhelmstr. 27, D-72074, Tübingen, Germany.

10:00 "Quantitative analysis of gene expression – a valuable tool in clinical immunology."
Giese T[1], Stallmach A[2], Zeier M[3] & Meuer SC[1]; [1]Institute of Immunology, University Hospital Heidelberg; [2]Dept. Gastroenterology, Catholic Hospital Essen-Nord; [3]Dept. Nephrology, University Hospital Heidelberg

10:20 – 10:45 **Coffee break**

"Real-time PCR is the most sensitive technique for biomolecular detection. Possibilities and Limitations in Research and in Clinical Diagnostics."

Mikael Kubista, Chalmers University of Technology and the TATAA Biocenter, Göteborg, Sweden (mikael.kubista@tataa.com)

Technical development in science usually moves forward in small steps. Existing technologies are refined and small improvements are constantly being made. But, perhaps once every 20 years, a new technology platform is developed that replaces old technology. This happened with real-time PCR. Real-time PCR technology has reached the ultimate sensitivity of detecting a single molecular copy of a nucleic acid molecule in a complex biological sample. It requires minute amounts of samples, making it possible to replace surgical biopsies with fine needle aspirates in routine testing, and in research study expression in rare cells and even parts of cells collected by laser microdissection. Advance probe technologies give selectivity to distinguish molecules that differ in a single position, and provide accuracy to determine DNA amounts with a precision of +/- 10-20 %. User-friendly instruments have become available that allow for very fast run times or high throughput. Even battery operated instruments for field use are available. The entire process from sampling to data analysis is becoming streamlined and automated to reduce experimental errors and eliminate artifacts. Use of hazardous chemicals, such as radionuclides and acryl amide, is totally eliminated. With these attributes, real-time PCR outperforms any alternative methods for nucleic acid quantification. In my talk I will summarize current state of the art of real-time PCR, providing several examples from our laboratory, discuss current possibilities and limitations, and also try to forecast how the field will develop.

"Laser capture microdissection and real-time PCR in Human Cancer."

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Quantitative determination of DNA sequences and gene expression levels offers a powerful approach for the comparative analysis of normal and diseased, especially neoplastic, tissues. Remarkable progress has been made in recent years in the development of techniques for assessing DNA copy number and gene expression at the mRNA level. However, a crucial factor for the reliability of the results obtained with these advanced techniques is the use of morphologically well-defined cell populations. Since the cell population of interest might constitute only a minute fraction of the total tissue volume, the problem of cellular heterogeneity has been a major barrier to the molecular analysis of normal versus neoplastic tissue. Thus, tissue microdissection represents one of the most promising techniques enabling the isolation of malignant (or even pre-malignant) cells with no or minimal contamination by non-neoplastic cells (Walch A et al *Histochem Cell Biol* 2001,115:269-76). Combining laser-assisted microdissection and quantitative real-time PCR and RT-PCR methods, we analysed tumour samples at different levels. *Detection of gene amplification*. Alterations in gene copy number are one of the most important causes of deregulated gene expression that represents the hallmark of neoplastic cells. The exact quantitative detection of gene amplification in solid tumors is often hampered by an admixture of non-neoplastic by-stander and stroma cells (Lehmann U et al. *Am J Pathol* 2000, 156:1855-64). To overcome this problem laser assisted microdissection of tumor cells with real-time PCR technology enables the highly reproducible exact quantification of minute amounts of nucleic acids. This approach can be applied to the study of frozen and paraffin-embedded tissue samples as well as circulating tumor cells collected by filtration (as already reported by Vona G et al. *Am J Pathol* 2000, 156:57-63). *Gene expression analysis*. Commonly applied mRNA extraction procedures are performed with homogenized tissue and RT-PCR measurements reflect an average expression value (Fink L et al. *Nature Medicine* 1998, 4:1329-33). Approaching this topic with the laser-microdissection technology requires the optimisation of some methodological steps such as suitable sample storage, fixation and staining procedures of the section before the isolation of the isotypic cells and efficient RNA extraction from the microdissected sample. Examples of applications of this approach will be

reported. In conclusion, increasing evidence shows that laser microdissection can represent a useful tool for establishing more specific molecular characteristics of pathological lesions in the presence of cellular heterogeneity and in particular in tumours.

"Microarray data of pooled samples compared to real-time-PCR of individual samples in African Children with Malaria."

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The differential susceptibility to severe malaria in infancy was recently assessed by our group based on microarray hybridization results of pooled RNA samples. To evaluate the pooling effect on these data, 16 selected genes were analysed by TaqMan real-time-PCR done on 84 individual samples. These 16 genes were selected because of their specific expression pattern in either mildly or severely affected individuals or because they appeared to be similarly regulated in both disease outcomes. Stabilized RNA was isolated from whole blood of 24 healthy African children, 30 children with uncomplicated malaria and 30 children with severe malarial anaemia. Real-time-PCR was done with 'assays-on-demand' kits on an ABI SDS 5700 (Applied Biosystems, Foster City, USA). In general, the higher the hybridization signals on the arrays, the better the agreement between real-time and microarray data. In 11 of 16 cases (70%) the results of the arrays could be confirmed. In five cases the specific expression pattern based on array data was not reproduced.
Seven of the confirmed expression profiles belonged to genes found stronger expressed during severe malaria; one gene denoted as severe-specific was not reproduced by the second assay. Three genes found up-regulated in mild and severe malaria were confirmed as well as one specific for mild malaria patients. Four genes that seemed to be stronger expressed regardless of the severity of the disease on the microarrays were not found equally up-regulated in individual uncomplicated and severe malaria samples with the real-time PCR.
This is most probably due to a generally higher heterogeneity of the RNA populations in the cells isolated from uncomplicated malaria patients. We propose that real-time-PCR is essential to verify microarray data and is a useful alternative for high throughput expression profiling on individual samples.

"Quantitative analysis of gene expression – a valuable tool in clinical immunology."

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Objective assessment of inflammatory disease activity, risk of relapse in patients in clinical remission as well as functional monitoring of immunosuppressive therapy are currently unsolved problems in clinical immunology. We established simple procedures to approach these challenges. Here we discuss two applications of real-time PCR in diagnostic procedures based on gene expression.

1. Classical markers for disease activity in patients with inflammatory bowl disease reflect either systemic inflammation or are based on scoring of subjective parameters. However, monitoring of inflammatory markers in mucosal biopsies is a simple objective laboratory guide of grading inflammation of the intestinal mucosa. In addition, using this method we could establish predictive markers for therapeutic responses and could identify patients that would benefit from anti-inflammatory remission maintenance. Such markers are important for risk-benefit assessment of immunosuppressive therapy.
2. Cyclosporine A (CsA) has improved patient and organ graft survival, but the dichotomy of benefit and toxicity remains an unsolved issue. CsA treatment is still monitored according to CsA pre-dose levels, which barely reflect its biological effects. Although the widely accepted measurements of CsA concentrations seem to work for the majority of patients, it is not clear, whether they reflect optimal functional immunosuppression. We have employed real-time PCR to measure directly the functional consequences of calcineurin inhibition, namely inhibition of transcriptional activities of NFAT regulated genes in peripheral blood. This method allows us to identify patients that are "over-suppressed" and might benefit from a reduced dosage of the drug. In a ongoing clinical study, using this method of monitoring, we could show that a lower dosage of CsA reduces nephrotoxicity without compromising the level of immunosuppression.

Session: qPCR Application in Microbiology & Virology

Chairs: U. Reischl & H. Nitschko

Lecture hall HS 14

10:45 Session introduction by U. Reischl & H. Nitschko

10:50 "LightCycler Applications in Diagnostic Bacteriology."
Udo Reischl, Institute of Medical Microbiology & Hygiene, Regensburg, Germany

11:20 "Genotyping and quantification of hepatitis C virus using fluorescent probes."
Hans Nitschko, Max-von-Pettenkofer Institut, LMU; Munich, Germany sponsored by Abgene

11:50 "Dissection of the retroviral life cycle using real-time PCR assays."
Klein D, Nosek D, Leichsenring B & Knapp E; Institute of Virology, University of Veterinary Medicine Vienna, Austria

12:10 "Real-time quantitative PCR assays for the detection and monitoring of pathogenic human viruses in immunosuppressed pediatric patients."
Suda Magdalena, Matthes-Martin Susanne and Lion Thomas; Div. Mol. Microbiology and Development of Genetic Diagnostics, Children´s Cancer Research Institute, Vienna

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

"LightCycler Applications in Diagnostic Bacteriology."

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We have developed and evaluated diagnostic LightCycler PCR protocols for a number of bacterial and fungal pathogens in the field of medical microbiology. They include, for example, the specific detection of enterodiarrheagenic *Escherichia coli* strains (Shiga toxin-producing *E. coli*, enterotoxigenic *E. coli*, enteroaggregative *E. coli*, enterophathogenic *E. coli*), methicillin-resistant *Staphylococcus aureus* (simultaneous detection of methicillin-resistance gene *mecA* and a *S. aureus*-specific marker within one capillary using the "*dual color option*"), *Legionella pneumophila*, *Mycoplasma pneumoniae*, *Chlamydia pneumoniae*, *Helicobacter pylori*, *Helicobacter pylori* clarithromycin-resistance testing (23S rDNA mutation detection by hybridization probes melting point analysis), *Helicobacter* spp., *Bordetella pertussis* and *parapertussis* (duplex assay using the "*dual color option*"), *Toxoplasma gondii*, *Corynebacterium diphteriae*. These parameters represent a subset of infections where a rapid, sensitive and specific detection of the causative pathogen is important for antibiotic treatment and the prognosis of an idividual patient. Especially in cases where clinical sample material can be obtained in a quantitative manner (e.g., blood, urine, CSF), quantitative LightCycler PCR may add a piece of valuable information for the treating physicians.
PCR primer and target sequences, which have been thoroughly evaluated in our routine diagnostic laboratory over the last decade, were adapted to the LightCycler. No seroius problems with respect to sensitivity and specificity of the amplification process were experienced. Since a sequence-specific confirmation of the identity of the amplification products is more and more considered as an essential prerequisite in diagnostic microbiology, we exclusively used the LightCycler "*hybridization probe*" format for product detection. The careful selection of suitable probe sequences within the amplicons in combination with the dual color option enabled us to distinguish even between closely related sequences and subtypes (e.g., specific detection of Stx-1 and Stx-2 and differentiation between subtypes of Stx-2 genes in enterohemorrhagic *E. coli*) within a single LC reaction capillary. With respect to microbiological practice, the simplicity, sensitivity and the simultaneous detection of "two parameters in a tube" are clear advantages of such novel assay concept.
Enhancing the reliability of the results by sequence-specific probes and simplifying the PCR workflow by a completely automated amplification and online detection procedure, the LightCycler system proved itself as a valuable tool for rapid diagnostic procedures in the environment of a routine microbiological laboratory setting. With the availability of the recently introduced LightCycler Instrument Vers. 2.0, the spectrum of diagnostic applications can be expanded and perhaps simplified by multiplexing.
Furthermore, we are currently evaluating the novel MagNAPure LC device for automated DNA extraction from bacterial & fungal pathogens and, more challenging, directly from clinical specimens.

"Genotyping and quantification of hepatitis C virus using fluorescent probes."

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The quantification of hepatitis C virus (HCV) RNA and the determination of the HCV-genotype are the two most important parameters in indication and monitoring of antiviral therapy. Several commercial assays are available for the analysis of either parameter, however no system permits fast genotyping and viral load measurement in parallel using only one analytical platform.
We developed a real-time PCR assay for the quantification of HCV RNA in clinical sample materials within a range of 10^2 to 10^8 IU/mL. The efficacy of nucleic acid isolation, reverse transcription and amplification is monitored by the addition of an internal positive control RNA to each individual sample prior to RNA extraction. Loss or reduction of the signal derived from this quantified *in vitro* transcribed control does indicate inhibition or competition during the isolation and/or amplification process. Comparison with different other HCV viral load measurement assays (COBAS Amplicor Monitor, bDNA) revealed excellent agreement of results and proved the wide linear range of this method.
In addition we defined a set of HCV genotype specific fluorescent probes which can be used to determine and differentiate between the in Europe most commonly detected HCV genotypes 1, 2, 3 and 4. These minor groove binder (MGB-) probes hybridize to a PCR amplified 150 nt fragment derived from the 5'-non-translated region of the viral genome. This type of analysis can be performed on the same microtiter plate that is used for viral load measurement on an ABI PRISM SDS 7700 "TaqMan" system. Genotyping results were confirmed by restriction fragment length polymorphism analysis and sequencing.
Thus both parameters, HCV genotpye and viral load, can be conveniently and reproducibly determined in parallel within 4 hours using only one analytical platform in one microtiter plate.

"Dissection of the retroviral life cycle using real-time PCR assays."

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The transduction efficiency of viral vectors can easily be monitored during pre-clinical trials by inclusion of marker genes (Klein et al. (1997) Gene Therapy 4, 1256-1260). However, the use of such marker genes has to be avoided in the final clinical gene therapy situation since their products often represent powerful immunogens and lead to the elimination of transduced cells. Thus it is not desirable to use such genes as markers in clinical settings, especially if the vector is applied *in vivo*. In these cases PCR based methods like the real-time PCR might provide a powerful tool to estimate biodistribution (Klein (2002) Trends in Molecular Medicine 8, 257-260). In order to investigate the accuracy and precision of this method, we have developed and tested a real-time PCR assay for the quantification of the enhanced green fluorescent protein (EGFP) gene and compared the results with transduction efficiencies estimated by FACS analysis (Klein et al. (2000) Gene Therapy 7, 458-463).
In order to improve these retroviral vectors, new real-time PCR assays for different steps of the retroviral life cycle have been developed. These assays enabled the identifcation of the impaired step and allowed the improvement of the retroviral vectors by 2-3 log decades (Hlavaty et al. (2004) Journal of Virology, in press).
Another important topic of retroviral vectorology is to minimize the risk of insertional tumorgenesis. In order to avoid this potential risk, targeted integration into the host genome would increase the safety of retroviral vectors and might provide a first step towards gene repair. The first step towards this long-term goal is the inhibition of the retroviral integration machinery without any impairment of earlier steps in the retroviral life cycle. Therefore we developed further real-time PCR and RT-PCR assays to follow the kinectics of the retroviral life cycle in the infected cell and compared the replication kinetics of several integrase mutants in contrast to the wild-type integrase.

"Real-time quantitative PCR assays for the detection and monitoring of pathogenic human viruses in immunosuppressed pediatric patients."

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Div. Mol. Microbiology and Development of Genetic Diagnostics, Children´s Cancer Research Institute, Vienna;
A panel of 23 real-time PCR assays based on the TaqMan technology has been developed for the detection and monitoring of sixteen different viruses and virus families including the human polyomaviruses (PoV) BKV and JCV, the human herpes viruses HHV 6, 7 and 8, the human adenoviruses (AdV), the herpes simplex viruses HSV 1 and 2, the varicella zoster virus (VZV), the cytomegalovirus (CMV), the Epstein Barr virus (EBV), the parvovirus B19 (PVB19), the influenza viruses (INF A and B), the parainfluenza viruses (PIV 1-3), the enteroviruses (ENV), and the respiratory syncycial virus (RSV). The test systems presented have a broad dynamic range and display high sensitivity, reproducibility and specificity. Moreover, the assays allow precise quantification of viral load in a variety of clinical specimens. The ability to use uniform PCR conditions for all assays permits simultaneous processing and detection of many different viruses, thus economizing the diagnostic work. Our observations based on more than 50.000 assays reveal the potential of the real-time PCR tests to facilitate early diagnosis of infection, to monitor the kinetics of viral proliferation and the response to treatment. We demonstrate that in immunosuppressed patients with invasive virus infections surveillance by the assays described may permit detection of increasing viral load several days to weeks prior to the onset of clinical symptoms. In virus infections, for which specific treatment is available, the quantitative PCR assays presented provide reliable diagnostic tools for timely initiation of appropriate therapy and for rapid assessment of the efficacy of antiviral treatment strategies.

Session: Normalization & Standardization

Chairs: T. Köhler & M.W. Pfaffl

Lecture hall HS 14

13:30 Session introduction by T. Köhler & M.W. Pfaffl

13:40 "Accurate normalization of gene expression using multiple internal control genes."
Jo Vandesompele, Center of Medical Genetics, Ghent University, Belgium

14:10 "Normalization genes for heart failure."
Kristin Brevik Andersson, Institute of Experimental Medical Research, Oslo

14:40 "Validation of housekeeping genes for normalising RNA expression."
Jim Huggett, Center for Infectious Diseases, London, UK

15:10 – 15:40 **Coffee break**

15:40 "Relative Gene Expression Studies using Multiplex Quantitative PCR on the Bio-Rad iCycler iQ Real Time PCR Detection System."
Hilary Srere, R & D Bio-Rad Laboratories, Hercules, CA, USA

16:10 "Standardized gene expression profiling and tumor prognosis."
Thomas Köhler, Roboscreen, Leipzig, Germany

16:50 "A Housekeeping-Gene Free Zone for Normalization."
Tania Nolan, Stratagene Europe, Amsterdam, The Netherlands

17:10 "Housekeeping gene expression in human seminoma and normal testicular tissue."
Neuvians TP, Sauer CG, Bleyl U & Grobholzer R; Pathologisches Institut, Universitätsklinikum Mannheim der Rupprecht-Karls-Universität Heidelberg, Deutschland

17:30 "Pitfalls in transfer of diagnostic duplex qPCR assays between technological platforms."
Tobias Ruckes, Artus GmbH, Hamburg, Germany

17:50 – 19:00 **Poster session**

19:00 – 22:00 **Bavarian Gala Dinner in the *„Oldest brewery of the world"* WEIHENSTEPHAN**
or alternatively

19:00 – 22:00 **Mediterranean Gala Dinner**

"Accurate normalization of gene expression using multiple internal control genes."

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Center for Medical Genetics, Ghent University Hospital, Belgium

With the advent of sensitive and accurate gene expression technologies such as real-time quantitative RT-PCR, the requirements for a proper internal control or so-called housekeeping gene have become increasingly stringent. Although many studies have reported that housekeeping gene expression can vary considerably, no study has systematically addressed the critical issues of using housekeeping genes, nor proposed an adequate workaround. In a recently published paper, we rigorously measured the expression level of 10 common housekeeping genes in 85 samples from 13 different human tissues*. Special attention was paid to select genes that belong to different functional and abundance classes, which significantly reduced the fact that genes might be co-regulated. We firmly demonstrated that the common practice of using a single housekeeping gene for normalization (such as beta actin, or glyceraldehyde-3-phosphate dehydrogenase), can result in erroneous normalization by at least a factor 3 in 25% of the tested samples. In order to address the expression stability of a given control gene in a series of tissue samples, we developed a conceptually novel and robust gene expression stability measure, and outlined an algorithm to determine the most stable and hence reliable housekeepers in a given tissue panel. The algorithm is based on repeated gene stability measurements and subsequent elimination of the least stable control gene.

To handle the large amount of calculations, a Visual Basic application for Microsoft Excel (termed geNorm) was developed and is freely available (more info on http://allserv.ugent.be/~jvdesomp/genorm/).

We also presented a procedure to determine how many control genes are required for reliable normalization. It turned out that 3 genes sufficed for samples with relatively low expression variation, but that other tissues or cell types required a fourth or fifth control gene to deal with the observed expression variation. Finally, we validated the expression stability measure and the geNorm algorithm on two levels. First, we showed that most non-specific expression variation (which should be completely absent after perfect normalization conditions) was removed when stable control genes (as determined by geNorm) were used for normalization, in contrast to the use of random housekeeping genes. Further validation based on publicly available microarray data demonstrated that our proposed normalization factor was equivalent to frequently applied microarray normalization factors.
In conclusion, we described and validated a procedure to identify the most stable control genes in a given set of tissue samples, and to determine the optimal number of genes required for reliable normalization of RT-PCR data. The presented strategy can be applied to any number or kind of genes or tissues, and should allow more accurate gene expression profiling. This is of utmost importance for studying the biological significance of subtle expression differences.

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"Normalization genes for heart failure."

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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 discussion of proper controls for comparing the mRNA expression level of specific genes between samples has therefore been revived.
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), □-actin and 18S ribosomal RNA have been the most frequently used reference genes.
In heart failure gene expression studies, GAPDH and 18S ribosomal RNA are the most commonly used normalization genes. In development of heart failure, the myocardium re-expresses fetal isoforms of ion channels and contractile structural genes, and undergoes a shift in metabolism from fatty acids to carbohydrates as energy source. We have observed a trend of decrease in GAPDH compared to 18S in left ventricle from failing heart, and a recent report showed that GAPDH expression was changed in electrically stimulated neonatal cardiomyocytes. The use of GAPDH as a reference gene may therefore be questionable.
We have evaluated the variability of 8 candidate normalization genes and compared them to GAPDH and 18S ribosomal RNA in a mouse post-infarction heart failure model. RNA was isolated from sham-operated and 6 week post-infarction hearts (three in each group). All RNA samples were quality checked on a Bioanalyser (Agilent) and by evaluation of 18s QPCR dilution curves. Both normalization genes and methods were evaluated by calculating the relative changes in commonly measured genes cited in the heart failure literature such as ANP, Serca2, NCX1 and phospholamban. The expression level of each gene was normalized to each of the 8 candidate normalization genes as well as GAPDH and 18S ribosomal RNA, or by comparison with a calibrator RNA sample (mix of sham and post-infarction hearts). Work in progress will be presented.

"Validation of housekeeping genes for normalising RNA expression."

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House keeping (HK) genes are traditionally used as a reference to control for error between samples. This practice is being questioned as it becomes increasingly clear that some HK-genes like GAPDH may vary considerably in certain biological samples. We used real-time RT-PCR to assess the levels of 13 HK-genes expressed in PBMC culture and whole blood from healthy individuals and those with tuberculosis. None of the commonly used HK-genes e.g. GAPDH were found to be suitable as internal references as they were highly variable (>30 fold maximal variability). Furthermore genes previously found to be invariant in human T cell culture also showed large variation in RNA expression (>34 fold maximal variability). Genes that were invariant in blood were highly variable in PBMC culture. Our data show that RNA specifying human Acidic Ribosomal Protein was the most suitable HK-gene for normalising mRNA levels in human pulmonary tuberculosis. Validations of HK-genes are highly specific for a particular experimental model and are a crucial component in assessing any new model.

"Relative Gene Expression Studies using Multiplex Quantitative PCR on the Bio-Rad iCycler iQ Real Time PCR Detection System."

Hilary Srere, R & D Bio-Rad Laboratories, Hercules, CA, USA

Real-time PCR is a powerful advancement of the basic PCR technique, in which starting amounts of RNA and

DNA can be accurately quantitated with appropriate fluorescent detection strategies and instrumentation. Proper experimental design is essential for studies involving relative or absolute comparisons between two or more genes amplified in the same tube, a technique known as multiplexing. Combining the techniques of reverse transcription PCR (rt-PCR) and multiplex real-time PCR (qPCR) is proving to be one of the most powerful tools in the modern researcher's arsenal to study relative gene expression in many diverse experimental systems. We will present data comparing the relative expression of several genes involved in the polyamine biosynthesis pathway, including ornithine decarboxylase (ODC) and S-adenysyl methionine decarboxylase (SAMDC), in the human thymus and prostate. In addition, guidelines for optimization of rt-PCR and multiplex qPCR experiments quantitating up to four gene targets in a single reaction will be discussed.

"Standardized gene expression profiling and tumor prognosis."

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Clinical decisions particularly at the treatment follow-up of virus diseases and hematological cancers are more and more based on quantitative molecular diagnostic results. Although practiced in quantitative clinical chemistry for a long time no comparable certified and stabilized calibrators with „set value" were available for molecular diagnosis until now. Based on more than 10 years of experience we created a new industrial standard: the "intelligent reaction tube". Intelligent tubes mean that common glass capillaries or plastic tubes required for either standardized nucleic acid purification or quantification are already precoated with defined amounts of distinct control DNA or RNA, respectively. They are manufactured by a novel technology covered by several patent applications. New properties like ready-to-use applicability, storage and delivery even at room temperature, highly reproducible data recovery, etc. are making this technology highly convenient for multicenter studies, routine laboratory scale and high-throughput molecular diagnostics.
Here we demonstrate results from a number of clinical studies concerning leukemias (AML, CML) and soft tissue sarcoma (STS) treatment using stabilized calibrators included in standardized real-time RT-PCR/PCR quantification protocols. Housekeeping gene-normalized gene expression data from already accepted markers like bcr-abl mRNA in case of CML and candidate genes such as apoptosis, signaling pathway and multidrug resistance genes were correlated to therapy success or progression-free survival in different cancers. By applying multivariate statistical analyses a number of independent predictors of unfavourable outcome already at the time of initial diagnosis such as high expression of bax and bad in AML, overexpression of survivin and mdm-2 in STS have been found.
Summarizing, the application of stabilized calibrators in standardized real time RT-PCR/PCR allows to obtain highly reproducible, comparable and statistical convincing results in different laboratories in the field of clinical diagnostics and prognosis evaluation for different cancers.

"A Housekeeping-Gene Free Zone for Normalization."

HA Lacey[1], T Nolan[2], S Greenwood[1], J Glazier[1] & CP Sibley[1] (tania.nolan@stratagene.com)

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There are clear differences in the rate of fetal growth through gestation, probably regulated via mechanisms of placenta transport control which includes the activity of the Na^+/H^+ exchanger (NHE) and Cl^-/HCO_3^- anion exchanger (AE). In order to correlate the presence of these transcripts with gestation, real time quantitative PCR (QPCR) was used to examine the changes in steady state levels of NHE or AE isoform mRNA between first trimester (when maternal blood begins to infuse the placenta) and full term. Total RNA from clinical samples was quantified using Ribogreen® staining. A constant amount of RNA was added to the reverse transcription (RT) reaction and in order to control for systematic variations in RT efficiency of different sample batches, an additional calibrator sample (derived from term placenta tissue) was included with each RT reaction. Relative to the calibrator, transcript levels of NHE1, AE1, AE2, and β actin showed no variation across gestation whereas NHE2 mRNA increased 18 fold ($p<0.001$). GAPDH mRNA also increased 3.5 fold ($p<0.05$) providing further evidence that it is inappropriate to use GAPDH as a housekeeping gene for normalisation of transcript levels in the placenta when examining gestational changes. Expression of all quantities relative to total input RNA and controlling for differences in RT efficiency by inclusion of a reference, calibrator sample, provides a simple, rapid analysis approach that avoids the requirement for reference to an internal normaliser or "housekeeping" gene.

"Housekeeping gene expression in human seminoma and normal testicular tissue."

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Housekeeping genes are commonly used as endogenous references in quantitative RT-PCR. Ideally these genes are constitutionally expressed by all cell types and do not vary under experimental conditions.

Evidences are growing that even widely used reference genes such as beta-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) can greatly differ in experimental groups. Tissue of 9 normal testis were gained from autopsy cases, and classical pure seminoma specimens were obtained from 22 patients. Real-time RT-PCR was used to examine the mRNA-expression of ubiquitin, beta-actin, GAPDH, 18S-ribosomal RNA (18S rRNA) and porphobilinogen-deaminase (PBGD). Ubiquitin, which serves for protein degradation, was down-regulated ($P < 0.01$) by a factor of 1.9 in seminoma. GAPDH, an enzyme of the carbohydrate metabolism, beta-actin, a constituent of the cytoskeleton, 18S rRNA and PBGD, an enzyme of the porphyrine metabolism, were up-regulated ($P < 0.001$) by a factor of 2.0 to 3.9 in seminoma. Beta-actin expression was down-regulated ($p < 0.05$) by a factor of 1.5 in angioinvasive pT2-tumour stages, when compared to pT1-seminomas. The other housekeeping-gene expressions were not significantly different in the two tumour stages, but tended to be lower in pT2-stages. A normalisation of the target gene data with up-regulated housekeeping genes would equalise or underestimate up-regulated data and overestimate down-regulated data. Even the median of the investigated housekeeping genes would still be significantly up-regulated in seminoma. We conclude that none of the investigated housekeeping genes is suitable for normalisation of the target gene RT-PCR data, but may be essential for tumour metabolism in human seminoma.

"Pitfalls in transfer of diagnostic duplex qPCR assays between technological platforms."

Tobias Ruckes, Artus GmbH, Hamburg, Germany

Quantitative real-time PCR has become a valuable tool for many applications both in diagnostic routine and research. A broad variety of real-time PCR instruments from different manufacturers feature different technical specifications to provide tools for a large number of applications. These platforms are diverse in throughput size, the design of their optical detection system, or the software algorithms for signal recognition. These differences have a critical impact on an assay's overall performance and, in particular, on its detection sensitivity. Consequently, the transfer of a diagnostic qPCR assay from one technological platform to another may be a complex and tedious task. In particular, this is the case for any multiplex assay as fluorescence channel availabilities and signal interferences on different instruments must be considered. The obligatory inclusion of an internal control in all NAT based diagnostic tests to monitor both a successful nucleic acid extraction and the potential presence of PCR inhibitors requires substantial assay modifications when adapted to a novel platform. These may include probe design, reaction composition, and quantification methods. On basis of two pathogen detection systems several aspects of assay adaptation to different platforms will be outlined in detail.

Thursday 4 March 2004

Session: New Approaches in qPCR & Automatisation

Chairs: C. Wittwer & L. Wangh

Lecture hall HS 15

08:30 Session introduction by C. Wittwer & L. Wangh

08:40 "Real time PCR in a Core Facility: Helping others to help themselves."
Deborah Grove, Pennsylvania State University, USA

09:10 "Determination of real-time PCR efficiency - An overview of different methods."
Michael W. Pfaffl, Physiology - Weihenstephan, ZIEL, Center of Food & Life Science-Weihenstephan Germany

09:40 "LATE-PCR and Allied Technologies for Amplification and Utilization of Single-stranded DNA."
Lawrence Wangh, Brandeis University, Boston, MA, USA

10:10 "Expression Profiling of Candidate Genes: Assays-on-Demand Gene Expression products based on TaqMan MGB chemistry."
Manohar Furtado, Applied Biosystems R & D - Applera Deutschland GmbH, Germany

10:30 – 11:00 **Coffee break**

11:00 "Establishment and the use of multiplex applications."
Böll Inga, Roche Applied Science, Penzberg, Germany

11:20 "Efficient non-linear analysis of kinetic amplification for quantification and automated results calling."
Martin Lee, BioGene Limited, Kimbolton, UK

11:40 "Quantitative PCR – a novel tool for protein quantification."
Andreas Kage[1], Wolfgang Henke[2], Heiko Witt[3], Claudia Dahmen[4] ; [1] Charité - Universitätsmedizin für Berlin, Institut für Laboratoriums-medizin und Pathobiochemie, [2] Charité - Universitätsmedizin für Berlin, Institut für Laboratoriumsmedizin und Pathobiochemie, [3] Charité - Universitätsmedizin für Berlin, Abt. für Pädiatrie, Augustenburger Platz 1, 13353 Berlin; [4] AptaRes AG, Im Biotechnologiepark TGZ 1, 14943 Luckenwalde, Germany

12:00 "PurAmp - a New Quantitative Method for Preparation, Synthesis, and Amplification of Both cDNA and Genomic DNA in a Single Tube."
Lawrence Wangh, Brandeis University, Boston, MA, USA

12:20 "A sensitive method for the quantitation of residual DNA using Alu based sequences and real-time PCR amplification."
Nussbaum O, Oppenheimer-Shaanan Y, Eren R, Dagan S, Zaubermann, A; XTL Biopharmaceuticals Ltd., Rehovot, Israel

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"Real time PCR in a Core Facility: Helping others to help themselves."

Deborah Grove, (dsg4@psu.edu)

Pennsylvania State University, USA

The tremendous utility of real-time PCR techniques has resulted in an increasing demand for this versatile and sensitive technology. Primary gene quantification, microarray and RNAi validation, zygosity testing, detection and/or quantification of pathogens, contaminants, transgenes, and SNPs are currently being performed using real-time PCR. Many existing core facilities are being asked to provide these services to their researchers and new facilities devoted to real-time PCR methodology are increasing. Investigators do not want to spend time developing a technique, rather they want to be able to come to an experienced person or facility that will do their assays for them or guide them through the intricacies and pitfalls of this technique using a shared instrument. Factors to consider in setting up and running a real-time PCR facility are numerous. Equipment selection, SYBR®green vs. specific probes, one-step vs two step, type of probes, automation, charges, level of service, throughput, etc. must be taken into account. Who will provide the template? What type of template? What are the appropriate controls? How to do the analysis? How do different cores handle these questions? "One size does not fit all" and the core facility must be as versatile and adaptable as the

"Determination of real-time PCR efficiency – An overview of different methods."

Michael W. Pfaffl (pfaffl@wzw.tum.de)

Physiology - Weihenstephan, ZIEL, Center of Food & Life Science-Weihenstephan Germany

The PCR efficiency has a major impact on the fluorescence history and the accuracy of the calculated expression result and is critically influenced by PCR reaction components. Different tissues exhibit different PCR efficiencies, caused by RT inhibitors, PCR inhibitors and by variations in the total RNA fraction pattern extracted. Efficiency evaluation is an *essential marker in gene quantification procedure*. Constant amplification efficiency in all compared samples is one important criterion for reliable comparison between samples. This becomes crucially important when analysing the relationship between an unknown sequence versus a standard sequence, which is performed in all relative quantification models. In experimental designs employing standardization with housekeeping genes, the demand for invariable amplification efficiency between target and standard is often ignored, despite the fact that corrections have been suggested. A correction for efficiency in relative quantification, as performed in efficiency corrected mathematically models, is strongly recommended and results in a more reliable estimation of the 'real expression ratio' compared to NO efficiency correction. Small efficiency differences between target and reference gene generate false expression ratio, and the researcher over- or under-estimates the 'real' initial mRNA amount. Therefore efficiency corrected quantification corrections should be included in the automation and calculation procedure in relative quantification models. The assessment of the exact amplification efficiencies of target and reference genes must be carried out before any calculation of the normalized gene expression is done. LightCycler Relative Expression Software, Q-Gene, REST and REST-XL software applications allow the evaluation of amplification efficiency plots.
Several methods are described in the literature to calculate real-time PCR efficiency:

A) Efficiency calculation from the slopes of the calibration curve, according to the equation: **$E = 10^{[-1/slope]}$**.

B) Efficiency calculation from the absolute fluorescence increase in linear phase of each logarithmic fluorescence history plot.
C) Efficiency calculation on the basis of all fluorescence data points (starting at cycle 1st up to the last cycle), according to a sigmoidal or logistic curve fit model.
D) Efficiency calculation from the fluorescence increase only in the exponential phase, according to a polynomial curve fit $\mathbf{Y_n = Y_0 (E)^n}$.

Which efficiency calculation method is 'the right one' and which one shows the realistic real-time PCR kinetic and thereby is highly reproducible has to be evaluated in further experiments.
We propose a computing method for the estimation of real–time PCR amplification efficiency. It is based on statistic delimitation of a beginning of exponentially behaved observations in real-time PCR reaction kinetics. PCR ground fluorescence phase, non–exponential and plateau phase will be excluded from the calculation process by separate mathematical algorithms. We validated the method on experimental data on multiple targets obtained on the LightCycler platform. The developed method yields results of higher accuracy than currently used method of serial dilutions for amplification efficiency estimation. The single reaction setup estimation is sensitive to differences of starting concentrations of the target sequence in samples. Furthermore, it resists subjective influence of researchers, and the estimation can therefore be fully instrumentalised automated.

"LATE-PCR and Allied Technologies for Amplification and Utilization of Single-stranded DNA."

WANGH, L, SANCHEZ, A, RICE, J, PIERCE, K (Wangh@Brandeis.edu)

Brandeis University, Department of Biology, Waltham, MA, USA

Symmetric PCR generates double stranded DNA products via exponential amplification, but the reaction plateaus when analyzed in real-time. Signal strength in such reactions can be low because the fluorescent probe has to compete with the reannealing of the product strands, and the final fluorescence intensity among replicate reactions is highly variable. LATE-PCR starts out as exponential amplification of a double-stranded product and then switches to sustained (nonplateauing) linear amplification of just one strand. Thus, LATE-PCR is an improved form of asymmetric PCR but, in contrast to conventional asymmetric PCR, is just as efficient as symmetric PCR. LATE-PCR achieves reliable asymmetric amplification by making use of several innovations in the design of primers. LATE-PCR also utilizes an improved thermal cycle in which the annealing of the two primers to their respective target strands takes place prior to primer extension, while hybridization of the probe to its accumulating single-stranded target takes place after primer extension. This change makes it possible, in turn, to detect accumulating target strands over a wider and lower range of temperatures using probes that have lower melting temperatures. Probes of this design, such as molecular beacons, are more allele-discriminating and exhibited decreased levels of background fluorescence. Collectively these improvements increase the strength and reliability of signals and minimize scatter among replicate reactions. The wider detection-temperature range of LATE-PCR also makes it possible to use combinations of probes to determine which of many possible variant-target sequences, such as mutant strains of an infectious agent, is actually present in a sample. LATE-PCR also yields single-stranded amplicons in sufficient quantity and purity to be used directly for DNA sequencing. Our progress toward construction of a single-tube "sample-to-sequences" system based on LATE-PCR will be discussed.
LATE-PCR is patent pending and available for licensing from Brandeis University.

"Expression Profiling of Candidate Genes: Assays-on-Demand Gene Expression products based on TaqMan MGB chemistry."

Manohar Furtado (furtadmr@appliedbiosystems.com)

Applied Biosystems (Applera Deutschland GmbH)

Ready-to-use Gene Expression Assays-on-Demand(tm) products for more than 30,000 human, mouse and rat genes are available online. These assays eliminate the major bottlenecks in using real-time PCR for gene expression studies involving many genes. We will introduce myScience, the new free online database and workspace, including graphical viewers of the human and mouse genome and the transcriptome, as well as information about assay locations in the genomic context. Features and search possibilities will be demonstrated.

"Establishment and the use of multiplex applications."

Böll Inga (Inga.Boell@roche.com)

Roche Applied Science, Penzberg

Multiple color detections in Real Time PCR is a field of growing interest in molecular biological laboratories as this technique provides the opportunity to generate several data within one approach. Multiplex Assays can be used to measure target gene and reference gene in one reaction which minimizes assay variation. Furthermore, the amount of biological material needed is reduced and the cost factor compared to separated assays is decreased.
However, designing multiplex reactions may require some optimization strategies to cope with difficulties such as cross complementarities, competition of parameters, and by products which can impair results.
In this study, we will show proven strategies that simplify the development of new multiplex assays. Additionally, we present first results that quantification of three DNA targets in one reaction besides different expression levels up to five orders of magnitude is possible.
Furthermore, we can show mutation detection with four different DNA targets in one reaction and provide solutions for the improvement of melting peak analysis.

"Efficient non-linear analysis of kinetic amplification for quantification and automated results calling."

Lee, M A, & Webster, B. BioGene Ltd, BioGene House, Harvard Way, Kimbolton, Cambs PE28 ONJ.
m.lee@biogeneresearch.co.uk

The analysis of real-time amplification curves for quantification usually involves determination of a cycle number value (Ct) at a set threshold. This threshold is determined either by an automated function related to the fluorescence yield with respect to background (automated fit), or is set manually by the user (threshold bar). In either case the method may be used to generate a standard curve for the determination of unknowns by interpolation. A linear correlation coefficient (r) for the Ct value with respect to the concentration of standards is often promulgated as a good indication of data quality. However, a linear fit for quantification is on the whole, a poor one given the variable efficiency of reactions across the broad dynamic range of possible target concentration, and the large differences in fluorescence yield that may occur in some samples. Here we discuss an overview of the current approaches and their limitations. A new kinetic approach is demonstrated for applications including quantification and automated results calling.

"Quantitative PCR – a novel tool for protein quantification."

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[3] Charité - Universitätsmedizin für Berlin, Abt. für Pädiatrie, Augustenburger Platz 1, 13353 Berlin
[4] AptaRes AG, Im Biotechnologiepark TGZ 1, 14943 Luckenwalde

Aptamers are oligonucleotides with high affinity binding capabilities to a target molecule isolated in vitro from a combinatory library of oligonucleotide candidates. In analytical techniques those aptamers are commonly used with conventional techniques such as enzyme enhanced colorimetric systems known from antibody based technology. The colorimetric systems signals indirectly the amount of solid phase bound ligands (antibody or aptamer) and require the conjugation of the enzyme with the ligand. Quantitative PCR is a well established method for quantification of oligonucleotides. The technique is characterised by a broad range of detection and high sensitivity.
Here we present a new application of quantitative PCR which enables a direct quantification of bound aptamer in a competitive analytical test system for human serum albumin (HSA). DNA-oligonucleotides (65-mers) were selected as aptamers from a combinatory library using HSA as target. For the analytical test HSA was covalently immobilised to the surface of PCR-tubes. Unlabelled DNA-aptamers and soluble HSA as standards and samples with unknown HSA concentrations were coincubated. Afterwards, unbound DNA-aptamers were eliminated by extensively rinsing with washing buffer. PCR reaction mixture was added and the PCR tubes were sealed. The quantitative PCR was performed for 35 cycles using SYBR Green 1 as intercalating fluorescent stain. For calculation of unknown HSA concentrations the standard curve of ct-values versus standard concentrations of HSA was interpolated.
The results show a relationship of the ct-values and the HSA concentrations of the standard samples over about four decades. The sensitivity depends on the quantity of DNA-aptamers used for co-incubation and was in the range of albumin concentration in body fluids.
The data indicate that quantitative PCR can be the read out for a competitive aptamer-based assay and enables a reliable method for quantification of target molecules with unmodified oligonucleotide-aptamers. We will also present concepts for a similar sandwich type aptamer-based assay.

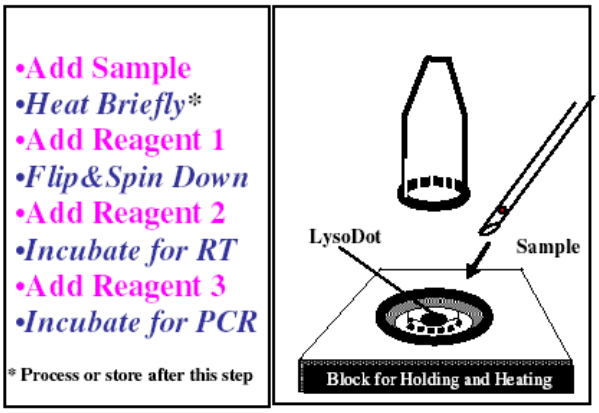
"PurAmp - a New Quantitative Method for Preparation, Synthesis, and Amplification of Both cDNA and Genomic DNA in a Single Tube."

WANGH, L, HARTSHORN, C, ANSHELEVICH, A (Wangh@Brandeis.edu)

Brandeis University, Department of Biology, Waltham, MA, USA

PurAmp is a novel single-tube method for preparing protein-free RNA from samples (even those as small as a single cell or less), reverse transcribing that RNA into cDNA, and then amplifying sequences within the cDNA via real-time PCR. Genomic DNA in the sample is also rendered protein-free and can either remain in the sample for amplification along with the cDNA, or can be removed by DNase treatment prior to reverse transcription. In either case, all of the steps are carried out in a single tube thereby minimizing losses, and each of the steps in the process has been fully optimized to permit quantitative detection over the range of 1-to-10,000 target molecules. In the application illustrated here a single mouse embryo comprised of 2-to~200 cells is placed onto a dried "LysoDot" previously spotted into the lid of a standard PCR tube. The sample is then heated briefly, after which point it can either be stored or processed immediately via the steps shown at the right. Non-transcribed genomic sequences in the sample serve as an internal standard for reaction reliability, as well as a means of counting the numbers of genomes present. In the case of transcribed genes the number of cDNA copies present in the sample is deduced from the CT value of the real-time reaction, after correcting for the number of genomic DNA copies amplified by the same primers. The utility of the PurAmp method will be illustrated for several genes in intact mouse embryos, single cells recovered from mouse embryos via laser ablation of the zona pellucida.

PurAmp is a patent pending technology that can be licensed from Brandeis University.



"A sensitive method for the quantitation of residual DNA using Alu based sequences and real-time PCR amplification."

NUSSBAUM O[1], OPPENHEIMER-SHAANAN Y[1], EREN R[1], DAGAN S[1], ZAUBERMAN, A[1] (onussbaum@xtlbio.com)

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Monoclonal antibodies derived from hybridoma or CHO cell lines have been used therapeutically to treat viral diseases and cancer. Production and purification of antibodies in cell culture may consist of residual genomic DNA as a potential contaminant. As a safety requirement, antibodies administered to humans should not contain residual DNA levels above 100 pg/dose. Consequently, a sensitive and reliable quantitative method for DNA determination is of utmost importance.

Objective: Development of a highly sensitive method for quantitation of residual specific DNA in cell cultures supernatants and in purified antibodies.

Method: The genomic DNA quantitative assay is based on PCR amplification of repetitive specific Alu sequences using real-time PCR and Taq-man MGB probes. Taq-man primers and probes were constructed against templates of either Human Alu-Sb subfamily consensus (HSU14568, 120 bp), which appear with 100% identity, at least 250 times in the human genome database or a Hamster cloned AluI-equivalent repetitive sequence (clone pNB137, 145 bp) that can detect transfected hamster DNA. DNA extracted from corresponding cell lines was used to establish a standard curve.

Results: DNA was extracted by DNAzol-BD and then subjected to real-time PCR using relevant primers and probes. The standard curve was sensitive to detect DNA ranging from 100 fg to 1 □g (250 fg/ml to 2.5 □g/ml). This assay was used to determine levels of residual DNA in purified monoclonal antibody preparations and in a supernatant of cell cultures. We found that prior to purification, supernatant derived from antibody producing cells contain about 10 □g DNA/mg IgG (3 □g/ml sup). Purified monoclonal antibodies were then tested for levels of residual DNA. These samples contain 500 fg DNA/mg IgG (500 fg/ml sample). Considering that the upper level of residual DNA should not exceed 100 pg/dose, therefore for this preparation, the maximal dose administered to humans should not be more than 200 mg IgG.

Conclusion: The sensitive assay that we have developed can be employed to determine residual DNA levels in clinical material, which should be used to treat humans.

Session: Transcriptomics & Expression profiling

Chair: M. Kubista

Lecture hall HS 15

13:40 Session introduction by M. Kubista

13:50 "Different approaches of data analysis in real-time amplification."
Thomas Kaiser, Corbett Research R&D, Australia sponsered by Pyrosequencing, Sweden

14:20 "Real-Time RT-PCR profiling of over 1,400 Arabidopsis transcription factors: Unprecedented sensitivity reveals novel root- and shoot-specific genes."
Czechowski T, Bari R, Stitt M, Scheibele WR & Udvardi MK; Max-Planck Institute of Molecular Plant Physiology, Am Mühlenberg 1, 14476 Golm, Germany

14:40 "PCR amplification efficiency, relative quantification and the analysis of alterations in cellular gene expression patterns."
Petrauskene OV, MacLean J, Wong a, & Furtado MR; Applied Biosystems, 850 Lincoln Center Dr. Foster City, CA, USA

15:00 "A rapid method to measure reactivity of brain regions upon stress exposure employing real-time qPCR analysis of activity regulated gene transcripts and calculation of recommended sample size."

Koya E[1], Spijkers S[1], Homberg J[2], Schoeffelmeer ANM[2], De Vries TJ[2], Smit AB[1]; 1 Department of Molecular and Cellular Neurobiology, Vrije Universiteit Amsterdam, The Netherlands; 2. Department of Medical Pharamcology, VU-Medical Center, The Netherlands

15:20 – 15:50 **Coffee break**

15:50 "Using real-time quantitative RT-PCR to validate a transcriptomics analysis advancing embryo-maternal communication."
Ulbrich S[1)], Bauernsachs S[2)], Rehfeld S[2)], Mallok S[3)], Prelle K[1)], Wenigerkind H[4)], Blum H[3)] ,Wolf E[2)] & Einspanier R[1,5)]
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16:10 "Multiplex BD QZyme Assays – a reliable real-time qPCR chemistry for analyzing the effects of gene silencing models."
Larsen Robert, BD Biosciences, Belgium

16:30 "Human Transcriptome Probe Library - detecting 37,000 genes with 90 probes."
Mouritzen P, Tolstrup N, Ramsing NB; Exiqon A/S, Bygstubben 9, DK2950 Vedbaek, Denmark

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"Different approaches of data analysis in real-time amplification."

Thomas Kaiser (thomas@corbettresearch.com)

Corbett Research R&D Australia sponsered by Pyrosequencing

Due to its sensitivity and high precision, real-time PCR has been rapidly adopted as a reference method for the determination of gene expression and gene copy number. In addition, it is faster and more reliable than Northern Blots and less subject to artefact than array hybridisation. Considerable effort has therefore been applied to methods of extracting reliable quantitative data from real-time fluorescence kinetics. Several publications have detailed methods for quantitation of absolute and relative gene expression and gene copy number from real-time PCR data and these will be discussed. Although widely used, the absolute quantitation method does require the creation, validation and maintenance of control samples for use as absolute standards. It also requires the input of identical (directly comparable) amounts of each unknown sample. More recently, methods for relative quantification have been developed which have the benefit of normalizing for sample input amount. Each relative quantification method requires satisfying a set of variables prior to final analysis, such as the selection of appropriate controls (calibrators) and normalisers (housekeepers). Relative quantitation can be divided into two groups; those requiring a standard curve (even at some stage in the validation experiment) and those not requiring a standard curve. Apart from the dual standard curve method, all other methods of relative quantitation determine the PCR efficiency and raise it to the power of the difference in take-off cycle (also known as the threshold cycle or crossing point) to determine relative target amounts. The exponential quality of this calculation makes the estimate of relative concentration very sensitive to errors in the determination of PCR efficiency. Therefore, methods that determine the reaction efficiency for individual samples rather than relying on standard curves provide more accurate results. Advantages and disadvantages of all these methods are presented in this talk.

"Real-Time RT-PCR profiling of over 1,400 Arabidopsis transcription factors: Unprecedented sensitivity reveals novel root- and shoot-specific genes."

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To overcome the detection limits inherent to DNA array-based methods of transcriptome analysis, we developed a real-time RT-PCR-based resource for quantitative measurement of transcripts for more than 1,400 Arabidopsis transcription factors (TF). Using closely spaced, gene-specific primer pairs and SYBR® Green to monitor amplification of double-stranded DNA, transcript levels of 83% of all target genes could be measured in roots or shoots of young Arabidopsis wild-type plants. Only 4% of reactions produced non-specific PCR products, and 13% of TF transcripts were undetectable in these organs. Measurements of transcript abundance were quantitative over six orders

of magnitude, with a detection limit equivalent to one transcript molecule in 1000 cells. Transcript levels for different TF genes ranged between 0.001-100 copies per cell. Whole-genome Arabidopsis Affymetrix-chips detected less than 63% of TF transcripts in the same samples, the range of transcript levels was compressed by a factor of more than 100, and the data was less accurate especially in the lower part of the response range. Real-time RT-PCR revealed 26 root-specific and 39 shoot-specific TF genes, most of which have not been identified as organ-specific previously. Finally, many of the TF transcripts detected by RT-PCR are not represented in Arabidopsis EST or MPSS databases. These genes can now be annotated as expressed.

"PCR amplification efficiency, relative quantification and the analysis of alterations in cellular gene expression patterns."

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Differences in gene expression patterns are a very good reflection of the functional status of a cell. Cellular gene expression patterns can be altered by the metabolic or developmental stage of a cell, disease processes, environmental stress and response to therapeutic agents. A major indicator of these changes is the measurement of transcript levels in cellular mRNA pools. Currently, the most sensitive and accurate method for detection and measurement of mRNA is the fluorescent quantitative real-time RT-PCR assay. Relative quantification (RQ) of transcript levels using real time PCR assays, without the need for generating standard curves for each assay, is a very useful tool for high throughput expression analysis of large numbers of genes involved in cellular regulatory networks. This is particularly useful for monitoring low abundance inducible transcripts and messages that are severely repressed in response to external stimuli.
In this abstract we outline our strategy to generate Taqman® Assays-on-Demand™ products that can be used to monitor transcript levels in human, mouse and rat cell extracts. Key features were that (i) all assays have near 100% PCR amplification efficiency for determination of RQ using the $2^{-\Delta\Delta Ct}$ method (ii) a high degree of specificity to be able to selectively amplify transcripts emanating from a targeted gene in the presence of transcripts emanating from paralogous genes. Important pitfalls and recommendations on how to accurately measure PCR amplification efficiency will be presented based on the analysis of over 1,000 assays. Reproducibility in RQ determinations will also be discussed.
Application of these assays in monitoring gene expression changes in the interferon signaling pathway in response to interferon-β induction and comparison the data in the literature will be presented. Data indicating the efficacy of these assays in monitoring siRNA based gene silencing and its effects on cellular networks will be discussed.

"A rapid method to measure reactivity of brain regions upon stress exposure employing real-time qPCR analysis of activity regulated gene transcripts and calculation of recommended sample size."

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2. Department of Medical Pharamcology, VU-Medical Center, The Netherlands

In many rodent studies, brain regions activated upon a stimulus are assessed through means of relatively time-consuming anatomical techniques such as in situ hybridization or immunocytochemical analysis involving one or few markers. These studies may take weeks to determine whether a brain region is reactive or not, and the lack of induction of one or few markers may not necessarily imply lack of reactivity. Here, we show that by employing multiple activity regulated genes (ARG's) well-known to be induced by various stimuli from *in situ* studies, we may answer relatively fast, if a brain region is reactive or not. Further, we show that each brain region has a unique gene expression pattern, with a reactive gene in one region not necessarily reactive in another. By finding which gene is highly regulated for a particular region allows one to tailor make an appropriate probe for further in situ hybridization studies rather than arbitrarily selecting a gene for all regions. Finally, we show that many of these activity regulated gene transcripts exhibit high inter-individual variability with a coefficient of variation (CV) of 30-70%. We provide the CV values on linear, non-log transformed values, as well as required amount of units to resolve particular levels of regulation for a selected set of CV values. Assuming a set CV for both experimental and control groups, for many of these markers a fold regulation of less than 1.4 fold may be difficult to resolve, as it may require n=16,12 respectively for experimental and control group for CV 30% and n=82,59 for CV 70%. These numbers are particularly useful when applying for grants/permission to order animals.

"Using real-time quantitative RT-PCR to validate a transcriptomics analysis advancing embryo-maternal communication."

ULBRICH S[1)], BAUERSACHS S[2)], REHFELD S[2)], MALLOK S[3)], PRELLE K[1)], WENIGERKIND H[4)], BLUM H[3)] ,WOLF E[2)] & EINSPANIER R[1,5)] (ulbrich@wzw.tum.de)

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The oviduct contributes to early reproductive events by providing an environment essential for the final transport, maturation and fertilization of the gametes. In an attempt to identify genes which are presumably involved in bovine embryo-maternal communication, a

transcriptomics approach was recently auspicious commenced (Bauersachs et al. 2003). A combination of subtracted cDNA libraries and cDNA array hybridization was applied to compare gene expression of ipsilateral and contralateral oviduct epithelial cells followed by a systematical study of differentially expressed genes at estrus and diestrus. 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.
Six heifers were synchronized and slaughtered at day 0 (n=3) or at day 12 (n=3) after they entered standing heat. Epithelial cells of ipsilateral oviducts were isolated, total RNA was extracted and candidate cDNAs were amplified using real-time RT-PCR (LightCycler®). Sequence-specific adjusted primers allowed a highly sensitive absolute quantification of even small changes in gene expression. Identical RNA samples as originally used for array hybridizations were analyzed enabling a direct comparison between both techniques.
The array hybridizations revealed 77 different cDNAs either up- or downregulated at estrus. The regulated genes or their products represented a variety of functional classes such as genes of the secretory pathway or involved in transcription regulation, cell-cell interaction or immune-related proteins or members of signal transduction pathways. Seven of these cDNAs were selected and their expression levels were determined by real-time RT-PCR. In each case, the results of the RT-PCR correlated markedly well with the results obtained by array hybridization ($r>0.92$). Moreover, the real-time RT PCR permits the absolute quantification of the abundance of every single candidate mRNA. It enables further comparative analyses in order to confirm the relevance of the results in a large number of individual samples.
In conclusion, the differential gene expression obtained by the holistic transcriptomics approach was clearly verified and quantified in more detail by real-time RT-PCR. A combination of both techniques seems most promising to rapidly identify and evaluate candidate genes involved in embryo-maternal communication.
Supported by the DFG Ei 296/10-1 and the Evangelisches Studienwerk eV.

"Multiplex BD QZyme Assays – a reliable real-time qPCR chemistry for analyzing the effects of gene silencing models."

LARSEN, ROBERT, BD Biosciences

(bobd_larsen@bd.com)

The BD QZyme Assay is a novel, readily multiplexed qPCR system based on a catalytically active DNA sequence, or DNAzyme, used for the quantification of specific cDNA and genomic DNA targets. Several advantages may be attributed to multiplex qPCR gene expression analysis: (1) optimized use of limited RNA/cDNA samples; (2) improved precision when measuring the expression level of two or three genes in relation to one another; (3) increased assay throughput; (4) and reduced cost per data point. We have effectively used multiplex BD QZyme Assays to analyze gene expression levels in several gene knockdown experiments. We will present quantitative gene expression level data for RNAi gene silencing targets p53 and NFkB, each multiplexed with housekeeping gene RPLO. In all cases, quantifying two or three genes simultaneously in one multiplexed reaction produced results nearly identical to each gene-specific assay run separately.

"Human Transcriptome Probe Library - detecting 37,000 genes with 90 probes."

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A novel approach to Real Time PCR assays employing short LNA enhanced 5'-nuclease probes - each capable of detecting numerous genes - is presented. Design of Real Time PCR assays for quantification of gene expression is a complicated and often laborious process. The time spent on design, oligonucleotide synthesis, and validation often limit the development and implementation of new assays. Ready-to-use assays are commercially available, but apart from being expensive these assays lack flexibility since each assay only target a single exon or exon splice site in each gene transcript, thus preventing the analysis of different splice variants. We would like to present a new concept, which by its universal nature avoids many of these inconveniences. A library consisting of only 90 dual labeled 5'-nuclease probes has been developed that will allow quantification of more than 99% of all 37,000 human gene transcripts submitted to the NCBI Reference Sequence Database. 79% of the gene transcripts are targeted at exon splice junctions thus facilitating design of intron spanning assays as a means to control erroneous quantifications caused by DNA contamination of RNA samples. In fact 39% of all exon splice junctions are targeted by at least one probe in the library, making the quantification of different splice variants an option. The unique universality of the probe library has been achieved by reducing the target sequence length of the 5'-nuclease probes from the classical 20-30 nucleotides to only 8 or 9 nucleotides. The use of these shorter probes allows selection of probe sequences that target frequently occurring genetags in human transcripts, which in turn result in a high degree of transcriptome coverage. To ensure compatibility with standard 5'-nuclease assay protocols, where annealing and extension is performed at 60°C, the duplex stabilizing DNA analogue LNA (Locked Nucleic Acid) has been introduced into the probes. The assay specificity rely on both the gene specific primers and the selected probe, thus avoiding the many adverse traits of unspecific assays such as SYBR Green assays. The web based assay selection tool will assist in selecting an optimal probe from the probe library and suggest a pair of PCR primers for any user defined target sequence. The high (>99%) coverage of the human transcriptome by the probes in the library will provide owners of the library with an instant access to quantitative PCR assays for almost any human gene. Examples will be presented showing applications of the probe library to quantification of human transcripts in different medical contexts ranging from cancer diagnostics to lipid metabolism and hypertension.

Session: Quality Assessment in qPCR
Chair: T. Bar
Lecture hall HS 15

16:50 Session introduction by T. Bar

17:00 "High Resolution Melting Curve Analysis."
Carl T. Wittwer, School of Medicine, University of Utah, USA

17:30 "Comparative Quality Assessment (CoQA) for real-time PCR."
Tzachi Bar[1] Neven Zoric[2], Anders Muszta[3] and Mikael Kubista[1, 2]; [1]Department of Chemistry and Biosciences, Chalmers University of Technology, Medicinargatan, [2]TATAA Biocenter, Medicinargatan, [3]Department of Mathematical statistics, Eklandagatan 86, 412 96, Gothenburg, Sweden

17:50 "An Italian external quality control program for quantitative PCR assay based on the use of TaqMan probes: results of a 42 laboratory survey."
Orlando C[1], Casini Raggi C[1], Pinzani P[1], Simi L[1], Verderio P[2], Marubini E[2], Pazzagli M[1]. [1]Clinical Biochemistry Unit, Department of Clinical Physiopathology, University of Florence, Italy; [2]Operative Unit of Medical Statistics and Biometry, European Institute of Oncology, Milan, Italy

18:10 "Data processing in real time PCR."
Larionov A.A., Miller W.R.; Breast Unit Research Group, Western General Hospital, Edinburgh, UK

18:30 – 19:00 **Poster session**

19:00 – 22:00 **Bavarian Gala Dinner in the *„Oldest brewery of the world"* WEIHENSTEPHAN**

or alternatively

19:00 – 22:00 **Mediterranean Gala Dinner**

"High Resolution Melting Curve Analysis."

Carl Wittwer, MD, PhD, Dept. Pathology, University of Utah, Salt Lake City, UT, USA
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High-resolution melting analysis of entire amplicons can be used for genotyping and sequence alteration screening. DsDNA dyes that are compatible with PCR at high concentrations allow closed-tube detection of heteroduplexes. After PCR amplification of heterozygotes, four duplexes are formed, two homoduplexes and two heteroduplexes. Each duplex has a characteristic Tm and the sum of all transitions can be observed through melting curve analysis. Using the high resolution melting instrument, HR-1, and the new DNA dye LCGreen I (Idaho Technology), single base changes can be detected in PCR products greater than 500 bps without probes. A systematic study of SNPs introduced into plasmids revealed a sensitivity of 100% in PCR products up to 400 bps. For genotyping SNPs, small amplicons are designed to bracket the SNP site to minimize the product size. Heterozygotes are easily detected because of heteroduplexes and homozygotes are identified by their Tm. Closed-tube SNP genotyping without probes or real-time PCR is demonstrated for common clinical targets. Mutation scanning is demonstrated for c-kit activating mutations in gastrointestinal stromal tumors, including SNPs, insertions and deletions. By amplifying highly polymorphic loci (e.g.) HLA genes, the sequence identity between two individuals can be easily determined with applications in tissue matching and forensics. High-resolution melting analysis goes far beyond a simple quality check of amplification products, and can completely replace probes in many genotyping applications.

"Comparative Quality Assessment (CoQA) for real-time PCR."

BAR T[1], MUSZTA A[2], ANDRADE-GARDA J.M.[3] & KUBISTA M[1,4] (tzachi.bar@molbiotech.chalmers.se)

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The exponential nature of PCR makes DNA quantification by real-time PCR sensitive to differences between the efficiencies of the compared samples. Therefore, the most common quantification methods require similar efficiencies for proper comparison. Based on this prerequisite, we suggest a Comparative model for Quality Assessment (CoQA) of real-time PCR. In CoQA, a set of experimental samples is considered of low quality if the ratio: variance of efficiencies of experimental samples / variance of efficiencies of high quality standard samples is > 3-4. Such standard samples may consist of purified PCR product, which shows the highest reproducibility in quantification. Using an F-test we tested the ratio between the variances of efficiencies of standard and experimental samples from 30 sets of 5 replicate each (same experiment on 5 animals). 8 sets of low quality quantification were identified. The variance of the DNA amounts quantified in the low quality sets were significantly higher ($P<0.01$) than the variance of the other sets. These findings suggest that the ratio of efficiencies variances could be used to draw attention of the real-time PCR user to suspicious samples.

"An Italian external quality control program for quantitative PCR assay based on the use of TaqMan probes: results of a 42 laboratory survey."

ORLANDO C[1], CASINI RAGGI C[1], PINZANI P[1], SIMI L[1], VERDERIO P[2], MARUBINI E[3], PAZZAGLI M[1]. (c.orlando@dfc.unifi.it)

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[3]Institute of Medical Statistics and Biometry, University of Milan, Italy

Increasingly, quantitative PCR techniques, based mainly on the use of fluorogenic probes and real-time detection of amplicons, are being used for the measurement of nucleic acids in research applications as well as in clinical laboratories. Therefore, as a matter of priority, external quality control programs (EQC) should be instituted that permit the evaluation of the analytical aspects common to the majority of molecular tests based on quantitative PCR. Since an EQC program cannot address all possible fields of application of this technique, a conventional quantitative scheme based on the measurement of a specific target in unknown samples is, for the time being, not practicable. However, several analytical aspects of real-time PCR are common to all procedures and independent of the specific target of interest. Therefore, we proposed a new EQC project targeting quantitative PCR, which started on May 2003, under the auspices of the Italian Society for Clinical Biochemistry and Molecular Clinical Biology (SIBioC).
The program involved 42 Italian laboratories that received: a. a cDNA sample to prepare external reference curve by serial 1 to 10 dilutions (from $2.5x10^7$ to $2.5x10^2$ copies); b. a primers-probe (FAM) mix, ready to use; three cDNA samples at different target concentration. The program incorporated two levels of controls: 1. the procedure for calculation of results; 2. the evaluation of quantitative results in 3 unknown samples. A comparative study was performed to evaluate analytical parameters used for sample calculation, i.e. standard curve parameters (slope, intercept), coefficient of correlation of standard curve, efficiency of amplification, threshold value, precision of triplicates, etc. In addition, the results obtained for each unknown sample were compared with the consensus median of all laboratories.
The analysis of 42 reference curves indicated good performances of linear fitting (consensus median: 0.997) and efficiency (95.6%) and elevated reproducibility among laboratories of Ct values of each standard dose (global CV% for 6 standard points: 1.27%). Reproducibility for unknown samples, evaluated on the respective absolute dose calculated on reference curve, indicated a dose-dependent variability with a CV ranging from 10.2% for the more concentrated sample ($3.6x10^6$ copies) to 36.7% for the less concentrated ($7.9x10^1$ copies). In particular, 11/42 laboratories were not able to detect the latter unknown sample and some differences in term of sensitivity emerged among different models of real-time instruments. In addition, the comparison of analytical parameters provided also a direct picture of the different approaches utilised by each participant for sample calculation.
In conclusion, this first phase of the project confirmed the importance and the practicability of the program. However, an overall evaluation of this EQC program indicates that the quality of real-time PCR assay methods already reached acceptable reliability and reproducibility, mainly connected to the use of dedicated instruments and optimised reagents.

"Data processing in real time PCR."

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Breast Unit Research Group, Western General Hospital, Edinburgh, UK

Transparent data processing is very important for interpretation of real time PCR (RT PCR) results. Several mathematical models have been developed to date. The choice of model depends on PCR design (*e.g.* standard curve / double delta designs), cycler features (*e.g.* availability of passive dye normalization, software features) and researcher preferences (*e.g.* favorable statistic methods). Today the software supplied with cycler often provides only a limited number of initial steps for analysis. In this case the choice of further mathematical procedure is at the researcher's discretion.

Here we outline our procedure for data generated by RT PCR with standard curve on each plate:

- the following order of steps is used to obtain crossing points from raw readings: smoothing, baseline subtraction, amplitude normalization and automatic selection of the best threshold (*i.e.* threshold that gives best coefficient of determination in the linear regression);
- mean crossing point from replicas and confidence limits of the mean are used for further

mathematical processing (regression, exponential function and normalization); at each step propagated confidence limits are traced;
- while using more than one referring genes they are summarized by the method of weighting.

To implement the procedure we developed software tool using VBA for MS Excel. It accepts raw data from CSV files generated by Opticon Monitor software (versions 1.4 and 2.01). Results are presented in form of the Excel workbook that contains numerical data and charts. All initial and intermediate data are easily available on separate sheets in the same workbook. Examples to illustrate these features will be presented from the analysis of expression of 6 genes in 42 breast cancer specimens.

While compiling the procedure the specific details of data processing varied notably in different software tools available today. Error propagation and their statistical evaluation varied most remarkably. Many of the details still remain to be standardized. The different options will be compared describing our procedure which is an attempt to find an optimal combination of commonly used and original steps aimed to produce easily perceptible and statistically valid results.

Friday 5 March 2004

Session: Nutrigenomics
Chair: H. Daniel
Lecture hall HS 14

08:30 Session introduction by H. Daniel

08:40 "Nutrigenomics: the road that leads to new insights into nutritional processes."
Hannelore Daniel, Molecular Nutrition Unit, ZIEL, Center of Food & Life Science Weihenstephan, Germany

09:10 "Nuclear Factors and Cytokines control TFFs down regulation in tumor cell lines of the digestive tract."
Baus-Loncar M, Dossinger V, Blin N, Gött P & Kayademir T; Institute for Anthropology and Human Genetics, Division of Molecular Genetics, University of Tübingen, Germany

09:40 "Differential regulation of the sodium-ascorbate co-transporters SVCT1 and SVCT2 expression in glutathione depleted CaCo-2 cells as assessed by functional analysis and quantitative real-time PCR."
Maulen NP[1], Kempe S[2], Nualart F[3], Bustamante ME[1] & Vera JC[2]; [1]Laboratory of Molecular Biology, Faculty of Medicine, Universidad Católica de la Santísima Concepción; [2]Department of Physiopathology, [3]Department of Cell Biology, Faculty of Biological Sciences, University of Concepción, Concepción, Chile

10:00 – 10:30 **Coffee break**

"Nutrigenomics: the road that leads to new insights into nutritional processes."

Hannelore Daniel, Molecular Nutrition Unit, ZIEL, Center of Food & Life Science Weihenstephan, Germany (daniel@wzw.tum.de)

Nutrigenomics seeks to provide a molecular understanding of how common dietary constituents and diets affect human metabolism and health by altering gene/protein expression and metabobolite profiles on basis of an individual's genetic makeup. Moreover, a gene guided approach is used to understand the function and nutritional role of a given protein in its physiological context. Gene-nutrient interactions are the paradigm for the interplay between the genome and the environment. We are just entering the era of functional genomics and there is no doubt, that molecular nutritional science is going to be of central interest as nutrients and other food components are key factors in affecting gene and protein activities. A wealth of the genetic information and novel techniques with high throughput capabilities provide fantastic new tools for nutrition research. Knowledge on the response of mammalian organisms to changes in their nutritional environment may be gathered at the mRNA and/or protein levels by expression-arrays, proteome analysis and high throughput metabolite profiling. With the toolbox of the "omics" nutritional science has become a new adventure in understanding the processes that make up human metabolism at all levels of its complexity.

Based on examples on the effects of individual nutrients (Zinc) and non-nutrient components (selected flavonoids) in mammalian cells and in model organisms are presented in which microarray analysis (and independent measures of changes in transcript levels) as well as proteome analysis tools are applied to understand the mechanisms by which these food components alter cell functions and metabolism.

Zinc is essential for the structural and functional integrity of cells and plays a pivotal role in the control of gene expression. To identify genes with altered mRNA expression level after zinc depletion, oligonucleotide arrays were used in human colon adenocarcinoma epithelial cell line HT-29 and in rats in experimental zinc deficiency. A low intracellular zinc concentration caused major alterations in the steady-state mRNA levels of several hundred genes at a threshold factor of 2.0. Northern blot analysis and/or real-time RT-PCR confirmed the array results for most of the selected targets. Genes identified as regulated based on microarray data encode mainly proteins involved in central pathways of intermediary metabolism (mainly protein metabolism). We also identified five groups of genes important for basic cellular functions such as signalling, cell cycle control and growth, vesicular trafficking, cell-cell interaction, cytoskeleton and transcription control. Proteome analysis identified a large set of proteins with altered steady state expression level that not necessarily showed the same kind or regulation on the transcript level. However, studies on changes in metabolite levels as predicted based on transcriptome profiling confirmed that clusters of proteins involved in the same pathways altered indeed substrate fluxes. Our findings in a homogenous cell population and in a complex animal model demonstrate that the molecular mechanisms by which cellular functions are altered at a low zinc status, occur via pleiotropic effects on gene and protein expression. The obtained pattern of zinc-affected genes/proteins may represent a reference for further studies to define the zinc regulon in mammalian cells.

"Nuclear Factors and Cytokines control TFFs down regulation in tumor cell lines of the digestive tract."

Baus-Loncar M, Dossinger V, Blin N, Gött P & Kayademir T (mbaus@hotmail.com)

Institute for Anthropology and Human Genetics, Division of Molecular Genetics, University of Tübingen, D-72074 Tübingen

The acute phase response is strictly connected with modulation of gene expression. Transcriptional control of many genes is mediated by binding of diverse transcription factors to cis-acting DNA motifs in the respective promoter sequence.We aimed at analyzing such regulatory elements for the trefoil peptide genes (TFF1, TFF2 and TFF3) coding for gastroprotective peptides. We assumed them to be regulated by the proinflammatory cytokines interleukin-1β (IL1β) and interleukin-6 (IL6), which trigger the transcriptional factors NF-κB and C/EBPβ. Following IL1β and IL6 stimulation, expression of *TFF* genes was analyzed in gastrointestinal cell lines HT-29 and KATO III by reporter gene assays using TFF promoter constructs and by quantitative real-time PCR. NF-κB and C/EBPβ were transiently co-expressed. We have functionally identified transcription factors NF-κB and C/EBPβ to inhibit transcription of human *TFF* genes. Down-regulation of *TFF* transcription is also observed by IL1β and IL6, suggesting crosstalk with or in response to the immune system. IL1β and IL6 caused a 3- to 11-fold reduction in *TFF* mRNA expression, displayed in real-time PCR. Down-regulation of intestinal trefoil factor TFF3 due to transcriptional repression by IL1β through NF-κB as well as by IL6 through C/EBPβ activation *in vitro* may reflect the situation *in vivo* and may contribute to ulceration and decreased wound healing during inflammatory bowel disease. Additionally, IL1 and IL6 over-expression in chronic gastritis may lead to mucosal damage and gastric carcinogenesis through transcriptional repression of TFF1 and TFF2.

Differential regulation of the sodium-ascorbate co-transporters SVCT1 and SVCT2 expression in glutathione depleted CaCo-2 cells as assessed by functional analysis and quantitative real-time PCR."

MAULÉN NP[1], KEMPE S[2], NUALART F[3], BUSTAMANTE ME[1] & VERA JC[2] (juvera@udec.cl)

[1]Laboratory of Molecular Biology, Faculty of Medicine, Universidad Católica de la Santísima Concepción, Concepción, Chile. [2]Department of Physiopathology, Faculty of Biological Sciences, University of Concepción, Concepción, Chile. [3]Department of Cell Biology, Faculty of Biological Sciences, University of Concepción, Concepción, Chile.

Vitamin C is an essential micronutrient required for the maintenance of normal human physiology. However, there is little information regarding the cellular mechanisms that control vitamin C transfer across the intestinal barrier and the bioavailability of the vitamin in humans. Vitamin C exists in two chemically distinct forms in human plasma, the reduced ascorbate ion form (ascorbic acid, AA), and the oxidized non-ionic form (dehydroascorbic acid, DHA), and plays an important biological role as an extraordinary reducing agent or intracellular antioxidant due its redox mechanism properties which mediates the protection of tissues from oxidative damage by scavenging free radicals.
We, recently reported in human colon carcinoma CaCo-2 cells, a human enterocyte-like cell line, the mechanism by which ascorbic acid is transported across the apical membrane of a mature polarized intestinal epithelium model. Our data showed that cell differentiation resulted in the upregulation and polarized expression of the sodium-ascorbic acid transporter SVCT1 (Maulén *et al*. (2003) *J Biol Chem* **278**(11):9035-41). However, remains unresolved whether vitamin C is processed intracellularly, or how it exits the basolateral membrane to be delivered to the rest of the organism. Several studies suggest the existence of a variety of mechanisms (enzymatic and non enzymatic), with the participation of glutathione (GSH), implicated in the maintenance of the intracellular antioxidant capacity. However, the molecular identity and the functional characteristics of those cellular mechanisms remains controversial.
In this context, we used CaCo-2 cells to study the effect of GSH depletion on the transport, accumulation and recycling of vitamin C. We obtained GSH-deficient cells by treatment with L-buthionine-(S, R)-sulfoximine

(BSO), an inhibitor of GSH synthesis, which allowed us to diminish de intracellular GSH about 100 times without affecting cell growth and viability. Transport, accumulation and recycling assays, together with competition and inhibition studies and quantitative real-time PCR analysis demonstrated that GSH participates in the accumulation of high intracellular concentrations of ascorbic acid (AA) and modulates the recycling of vitamin C in CaCo-2 cells. On the other hand, GSH depletion regulates the expression of glucose (GLUTs) and sodium-ascorbate (SVCTs) transporters, affecting directly the capacity of these cells to obtain the oxidized and reduced form of the vitamin. Specifically, we present data indicating that GSH depleted CaCo-2 shows differential regulation of SVCT1 and SVCT2 expression in response to the pro-oxidant conditions generated by GSH depletion. FONDECYT 3990007 y 1020451.

Session: Food Hygiene & GMO

Chair: H.H.D. Meyer

Lecture hall HS 14

10:30 Session introduction by Heinrich H.D. Meyer

10:40 "Real-time Detection and Quantitation of Genetically Modified Soy."
Babette Fahey, MJ Research Inc., Waltham, MA, USA

11:10 "Detection and quantitation of GMO in official food and feed control."
S. Pecoraro, Bavarian Health and Food Safety Authority Oberschleißheim, Germany

11:30 "CaMV virus detection with Real-time PCR – identification of false positive results in 35S screening for genetically modified organisms (GMOs)."
Cankar K, Gruden K, Tusek M, Toplak N, Ravnikar M, Žel J; National Institute of Biology, Department of Plant Physiology and Biotechnology, Večna pot 111, 1000 Ljubljana, Slovenia

11:50 "Finding the traces - real-time PCR assays for quantitative and qualitative detection of animal DNA in food and feed."
Bruns U, Müller M, Steinbohrn R & Müller S; Institute of Animal Breeding and Genetics, Veterinary University Vienna, Austria

12:10 "qPCR and Small Grain Cereals: Species and Transgene Detection."
Terzi V[1], Shewry PR[2], Stanca AM[1], Faccioli P[1]; 1 Istituto Sperimentale per la Cerealicoltura, Via San Protaso 302, 29017-Fiorenzuola d'Arda (PC) , Italy;: 2 IACR-Long Ashton Research Station, Long Ashton, Bristol BS18 9AF, UK

"Real-time Detection and Quantitation of Genetically Modified Soy."

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In this study, real-time quantitative PCR was used to detect and quantify genetically modified soy in soy-containing foods. Two different protocols were developed: the first was a one-color assay that used the double-stranded DNA binding dye SYBR® Green I to detect both the Roundup Ready® sequence and an endogenous reference sequence; the second was a two-color assay that used differentially labeled TaqMan® probes to detect the Cauliflower Mosaic Virus (CaMV) 35S promoter sequence (the promoter used to express the transgene) and an endogenous reference sequence. The amount of genetically modified soy was quantified by interpolation against a standard curve generated from a set of certified reference standards. The methods presented here have several attractive features, including: they require only small amounts of starting material; they are suitable for raw and processed foods; and they are adaptable for other genetically modified crops.

"Detection and quantitation of GMO in official food and feed control."

S. Pecoraro, Bavarian Health and Food Safety Authority Oberschleißheim, Germany
(sven.pecoraro@lgl.bayern.de)

In 2003, six principal countries grew 99 % of the global genetically modified (GM) crop area of 67.6 million hectares. The USA grew 42.8 mill. has (63 % of the

global total) followed by Argentina with 13.9 mill. has (21 %), Canada 4.4 mill. has (6%), Brazil 3 mill. has (4 %), China 2.8 mill. has (4 %) and South Africa 0.4 mill. has (1 %). Specific genetic modifications of these plants are predominantly newly introduced genes for the production of either bacteria derived (*Bacillus thuringiensis)* toxins against certain pests and/or the tolerance of nonselective herbicides (BASTA™, Roundup™), mediated by genes from microbial and plant sources. The four commercially most extensively grown transgenic plants are soybean, maize, cotton and canola occupying 61 %, 23 %, 11 % and 5 % of the global GM area, respectively. In the USA 80 % of the grown soybean is GM, in Argentina it is almost 100 %. In Europe there is no commercial cultivation of GM plants until now with the exception of Spain (30,000 has), but there is import and processing for the food and feed industry. A GMO can be put on the European market if it is approved and labelled. In 2003 two European regulations [(EG) 1829/2003 and (EG) 1830/2003] came into force which will expand existing regulations on GMO and which will be applicable in spring 2004. They deal with the authorisation of GM food and feed and with the traceability and labelling of GMO and GM food and feed products. According to these legislations there is a liability of labelling any food or feed that is a GMO (e.g. tomato, corncob), that contains or consist of a GMO (e.g. yoghourt with GM *Lactobacilli*) or that is produced from or contains ingredients produced from a GMO (e.g. oil from GM canola). In contrast to existing regulations labelling is required if there is direct application of GMOs during processing of food, feed or ingredients regardless of the analytical feasibility to find GM DNA sequences in the product. The regulation for traceability therefore states the liability to transmit all data between operators concerning the application of a GMO throughout the placing on the market of a product with the help of a unique identifier. A threshold of 0.9 % of an approved GMO related to an ingredient will be tolerated without labelling (present value: 1 %) if such traces are proved to be adventitious or technically unavoidable. For non approved GMO there is a zero tolerance.

Enforcement laboratories routinely investigate food samples for the presence of GMO. Each sample is tested qualitatively by amplifying plant specific DNA sequences by PCR. A second PCR test usually screens for GM specific DNA sequences (e.g. sequence of the GM DNA construct). If there are positive results in both cases the relative amount of GM DNA in the sample is determined by Real-time PCR. In 2003 the Bavarian Health and Food Safety Authority analysed 656 different food samples that contained soybean (374) and/or maize (293). Soybean containing food: 44 out of 374 samples (11.8 %) were qualitatively tested positive for GM Roundup Ready™ soybean. 37 samples (84.1 %) were determined to have a GM DNA content of lower than 0.1 %. Four samples showed GM DNA values lower than 1 % (9.1 %). In one case (2.3 %) there was a value above 1 %. Maize containing food: 39 out of 293 (13.3 %) samples showed positive results for GM DNA. In 37 cases (94.9 %) the GM DNA content was lower than 0.1 %. Two samples could not be quantified. By the new regulations feed is treated like food in terms of authorisation, traceability and labelling. Hence GM feed will also have to be labelled if it contains GM material. In 2003 fifty feed samples containing soybean, maize or canola were investigated. The highest levels of GM DNA were found in soybean containing feed. Fourteen out of 20 samples (70 %) were tested positive. Quantitation by Real-time PCR revealed GM DNA values of 1.3 to 100 %.

"CaMV virus detection with Real-time PCR – identification of false positive results in 35S screening for genetically modified organisms (GMOs)."

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For the production of food, the EU has approved products from GM soybeans, corn and rapeseed and had adopted labelling of all products that contain GMOs in concentration above 0,9% in order to guarantee the consumer's choice between GM and non-GM products. Screening of samples is performed using primers for regulatory sequences introduced to different transgenic plants of which primers for 35S promoter from Cauliflower mosaic virus are predominantly used. In virus infected plants or samples contaminated with plant material carrying the virus false positive results can occur. A system for Real-time PCR was designed that allows recognition of virus coat protein in the sample, thus allowing us to differentiate between transgenic plant and virus-infected plant. Efficiency of PCR reaction, limit of detection and quantification, range of linearity and repeatability of the assay were tested. Detection system was tested using 8 different CaMV virus isolates. No cross-reactivity was detected with DNA of 4 isolates of closely related Carnation etched ring virus. All virus samples were examined under electron microscope and presence of virus particles was confirmed. Primers do not amplify plant DNA neither from approved GM plants nor from different species of Brassicaceae or Solanaceae that are natural hosts of CaMV. We have evaluated the assay for different food matrices by spiking CaMV DNA into DNA from food samples and have successfully amplified CaMV from all samples. Presence of the virus was also confirmed in rapeseed samples from routine GMO testing, that were positive in 35S screening assay.

"Finding the traces - real-time PCR assays for quantitative and qualitative detection of animal DNA in food and feed."

BRUNS U, MÜLLER M, STEINBORN R & MÜLLER S (ute.bruns@i122server.vu-wien.ac.at)

Institute of Animal Breeding and Genetics, Veterinary University Vienna, Austria

Tools for quantitative determination of species-specific material are desired to control legal restraints for composition of food and feed. Several methods have been applied in order to find meat and bone meal (MBM) contaminations in feedstuff since the BSE crisis and the subsequent ban of MBM from feedstuff intended for ruminants. We designed assays based on mitochondrial DNA sequence information to universally detect mammalian and poultry derived DNA on the one

hand and additionally species-specific assays for cattle, horse, pig, chicken, turkey and mouse. Quantitative estimation of sample content was based on standard DNA extractions of either fresh or autoclaved (133°C, 3bar, 20min as mandatory by EU laws for MBM) muscle material of animal species and serial dilutions of the isolate. Assays were tested for specifity, target quantitation precision in presence of nontarget DNA either at equal amounts or in excess and of different origin (other animal species or feedstuff i.e. plant DNA). Errors for quantitation are due to specific mitochondrial DNA content of animal material, DNA extraction yield, PCR inhibitory or enhancing effects of nontarget DNA from total sample extraction and between-PCR-run differences. Therefore we applied a confidence interval approach using standard curves derived from independent extractions and serial dilutions in different runs. Hence, quantitative estimates represent a range but not a discrete value. However, this approach rather than calculating a single value appears more self-evident due to the many precarious factors and assumptions intrinsic to these analyses.
This project was financially supported by the Bundesministerium für Soziale Sicherheit, Generationen und Konsumentenschutz, Austria.

"qPCR and Small Grain Cereals: Species and Transgene Detection."

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The introduction of real time PCR technology has significantly improved and simplified the quantification of nucleic acids that is required in several plant science applications. We have applied this technology to the traceability of small grain cereals species and transgenes. Five cereal species (bread and durum wheat, maize, rice and barley) assure directly or indirectly 2/3 of world human food consumption and the cereal composition of specific food is always a key factor in the quality and safety of the final product. Pasta made from durum wheat is considered superior in several qualitative aspects to that manifactured from bread wheat or a mixture of the two species and consequently Italian law prohibits the manifacture of pasta for sale in Italy containing more than 3% bread wheat. Starting from seed storage protein sequences characterisation, we have developed an analytical systems based on real time PCR for *Triticum* species-specific detection and quantitation of bread wheat contamination in semolina and pasta (Terzi et al., 2003a).

Quality of wheat for food is mainly related to gluten content: structure and properties of wheat seed storage proteins play in fact a major role in grain utilization and nutritional quality. One group of gluten proteins, the high molecular weight (HMW) subunits of glutenin, appear to be particularly important in that they form an elastomeric network in dough that is associated with good breadmaking quality. Consequently, wheat transformation has been used to manipulate HMW subunit composition by adding genes for new subunits or increasing the copy numbers of genes for endogenous subunits, giving new GM wheat genotypes. A TaqMan PCR approach for the detection of a genetically modified durum wheat genotype has been developed (Terzi et al., 2003b): the target sequence has been identified inside the construct used to obtain this transgenic wheat that consisted of a 5' flanking region of the wheat *Glu-1D-1* gene driving the expression of 600 bp of the wild type tobacco *rab1* coding region. Moreover, in GM wheats the determination of transgene copy number is important for studies of the stability and inheritance of transgenes and their effects on grain functional properties. We have correlated the results obtained in term of transgene copy number in T1 bread wheat lines transformed to express 1Dx5 subunit through a quantitative real time PCR approach with the transgene expression levels in seeds. The aim of the work has been to evaluate real time PCR approach as alternative one to Southern analysis for rapid assisted selection of transformed genotypes (Terzi et al., submitted).

Cereal composition determination can be a relevant task even from a security point of view. In fact, for example, safety principles demand that in food for coeliac consumers the possible contamination of specific cereals must be accurately determined. Cereal species, for which the existence of proteins toxic for people affected by coeliac disease has been demonstrated, include wheat, rye, barley and the wheat-rye cross triticale. Consequently, we have developed PCR primers and a TaqMan probe for qualitative and quantitative detection of *Secale cereale* and *Triticosecale* in raw materials and processed food (Terzi et al., in press). The primers and probe are based on secalin and EST sequences publicy available and were tested for their species-specificity on a panel of grass genotypes and food.

Terzi, V., Malnati, M., Barbanera, M., Stanca, A.M., Faccioli, P. (2003a) Development of analytical systems based on real-time PCR for *Triticum* species-specific detection and quantitation of bread wheat contamination in semolina and pasta, *Journal of Cereal Science* 38(1) 87-94.

Terzi, V., Ferrari, B., Finocchiaro, F., Di Fonzo, N., Lamacchia, C., Napier, J., Shewry, P.R. and Faccioli, P. (2003b) TaqMan PCR for detection of genetically modified durum wheat. *Journal of Cereal Science* 37:157-163.

Terzi V., Infascelli F., Tudisco R., Russo G., Stanca A.M., Faccioli P. Quantitative detection of *Secale cereale* by real time PCR amplification. In press on *LWT, Food Science and Technology*.

Session: Detection methods
Chair: D. Whitcombe
Lecture hall HS 15

08:30 Session introduction by D. Whitcombe

08:40 "Scorpions- Application in Genotyping and Real time PCR."
David Whitcombe, DxS Genotyping, Manchester, UK

09:10 "Genotyping of SNPs via Fluorescent Melting Curve Analysis."
Thomas Fröhlich, Roche Diagnostics R & D LightCycler Development Group, Penzberg, Germany

09:40 "Real-time PCR genotyping using strand displacement probes."
Li Q, Cheng J, Zhang Y, Molecular Diagnostics Laboratory, Xiamen University, China

10:00 – 10:30 **Coffee break**

10:30 "LUX Fluorogenic Detection System and other new approaches in qPCR."
Debra Nickson, Invitrogen Europe

11:00 "LNA probes, a new tool to enhance your real Time QPCR applications."
Khalil Arar, Proligo SAS, Paris, France

11:30 "Novel amplification and detection chemistries for real-time PCR."
Dirk Löffert, Qiagen, Hilden, Germany

12:00 "Highly sensitive analysis of allele-specific gene expression by MALDI-TOF MS."
Stephen Sharp, Sequenom, Inc., San Diego, Germany

Closing of the Symposium
Lecture hall HS 14

12:30 - 13:00 "Closing of the Symposium."
Michael W. Pfaffl & H. H. D. Meyer

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

"Scorpions- Application in Genotyping and Real time PCR."

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We have developed a probe system called Scorpions in which the probe is attached to one of the PCR primers. The hybridization of the probe to its target is thereby converted to a unimolecular rearrangement event with zero order kinetics.
The consequences of this mechanism include:

- Concentration independent hybridization- efficient signalling throughout the PCR
- Favourable thermodynamics and kinetics generates high, robust signals
- Quenched probe formats lead to low baseline signals
- Instantaneous hybridization kinetics facilitates rapid PCR
- Reliable probe design due to predictable folding of probe and amplicon
- Improved hybridization characteristics allow shorter, more discriminatory probes.

We will present data illustrating these unique features and will demonstrate their benefits in challenging real time and genotyping applications.

"Genotyping of SNPs via Fluorescent Melting Curve Analysis."

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(thomas.froehlich@roche.com)

Roche Applied Science, Penzberg, Germany

Single nucleotide polymorphisms (SNPs) are the most abundant form of genetic variation, accounting for more than 90% of all differences between individuals. In recent years, genotyping of SNPs has become a key technology for genetic studies in industrial and public research with major impact on genome mapping, pharmacogenetics studies, drug discovery and population genetics. One of the next important steps, e.g. towards understanding and curing complex traits, is the establishment of genotype-phenotype correlation due to gene specific variations.
Full exploitation of available genomic information requires technologies capable of performing vast numbers of SNP analyses, as large numbers of individuals have to be screened for a large number of markers. Currently only few genotyping techniques can cope with all resulting demands concerning sample throughput, flexibility, accuracy, automation and cost-effectiveness.
With the LightTyper genotyping system we have introduced a platform capable of analysing thousands of individual samples per day. A straight-forward workflow concept is combined with dynamic software modules for run control and allele calling.
LightTyper based SNP genotyping is carried out via post-PCR, high resolution melting curve analysis of dye-labelled gene-specific oligonucleotide probes. Genomic target sequences are amplified in a standard thermalcycler in 96- or 384-well microtiter plates. After PCR-amplification plates are directly transferred to the LightTyper instrument where genotypes are read out within only 10 minutes. One major advantage of the workflow is that it is completely homogenous: all reagents are already introduced during PCR set-up, there is no need for further manipulation or sample purification prior to LightTyper analysis. Allele calling is performed automatically by comparing each sample to representative standards. Standard genotypes can be from within the run or imported from other files. Samples and genotypes can be further grouped allowing multiple-allele detection on few or many samples.

“Real-time PCR genotyping using strand displacement probes.”

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The molecular diagnostics laboratory, Xiamen University, China.

Simple and reliable genotyping technology is a key to success for high-throughput genetic screening in the post-genome era. Here we developed a new real-time PCR genotyping approach that uses displacement hybridization-based probes. One distinct feature of this method is that the probes designed with maximal specificity also have the greatest detection sensitivity. Furthermore, the specificity of strand displacement probes can be simply assessed through denaturation analysis before genotyping was implemented. The ease in design, the simple single-dye labeling chemistry, and the capability to adopt degenerated negative strands for point mutation genotyping make the strand displacement probes both cost-effective and easy in use. We analyzed the single C282Y mutation in the human hemochromatosis gene with this method. Accurate genotyping could be achieved with a single reaction. We went further to apply this method to simultaneous genotyping of five different types of mutation in the human β-globin gene. Totally 62 human genomic DNA samples with nine known genotypes were accurately detected and 32 random clinical samples were successfully screened. A blind study using 120 human genomic samples was also conducted and showed full agreement with the dHPLC and reverse-blot method. We believe this method with its combined merits of reliability, flexibility, and simplicity will be widely used for routine clinical testing and large-scale genetic screening.

"LUX Fluorogenic Detection System and other new approaches in qPCR."

Debra Ann Nickson PhD, European Product Manager, Invitrogen debra.nickson@invitrogen.com)

LUX™ (Light Upon eXtension) primers (Invitrogen) are an easy to use, highly sensitive, and efficient method for performing real-time quantitative PCR and RT-PCR. LUX™ Primers combine high specificity and multiplexing capability with simple design and streamlined protocols. They do not require any special probes or quenchers and are compatible with melting curve analysis of real-time PCR products, allowing the differentiation of amplicons and primer dimer artefacts by their melting temperatures. Due to their flexibility in use and lower cost LUX™ primers are suitable for high throughput applications. Each primer pair in the LUX™ system includes a fluorogenic primer with a fluorophore attached nearby its 3’end, as well as a corresponding unlabeled primer. The fluorogenic primer has a short sequence tail of 4-6 nucleotides on the 5’end that is complementary to the 3’end of the primer. The resulting hairpin secondary structure provides optimal quenching of the fluorophore . When the primer is incorporated into the double-stranded PCR product, the fluorophore is dequenched and the signal increases by up to 10-fold. LUX™ primers have been used in real-time PCR and RT-PCR to quantify 100 or fewer copies of a target gene in as little as 1 pg of template DNA (or RNA); they have a broad dynamic range of 7-8 orders. Multiplex reactions can be performed using separate FAM and JOE-labelled primer sets to detect two different genes in the same sample. Real-time RT-PCR using fuorogenic LUX™ primers has been shown as a reliable and effective method for quantifying several transcripts in neural stem cells; QRT-PCR was performed to determine the expression patterns of various transcripts in samples of pluripotent mouse P-19 stem cells. The P-19 cells were used because they transform into neuron-like cells upon retinoic acid treatment. The expression of neural and stem cell genes, including GLUR1, GABA-B1a, NMDA1, GAP-43, ChAT, BDNF, nestin, BMP-2, BMP-4, and EGR1, was increased, approximately 10- to 1000-fold, during the course of differentiation from 0 to 11 days after induction with retinoic acid. Quantification was performed both for each single gene and in duplex using GAPDH as a reference gene. Results of quantitative PCR utilizing dual LUX™ primer pairs were similar to quantitation by single LUX™ primers and to results derived by using an alternate method for qRT-PCR, the 5’-nuclease probe assay. Other detection methods, such as TaqMan™ or molecular beacons, involve fluorescence quenching by energy transfer between dye and quenching moieties in

dual-labelled oligonucleotides. These FRET-based oligonucleotides can provide a high signal to noise ratio in homogeneous assays. However, the addition of a separate probe to the PCR reaction increases the complexity of the reaction kinetics making optimisation more challenging. Also LUX™ primers in their hairpin conformation can actually enhance the specificity of the PCR by helping to prevent primer dimers and mispriming.

"LNA probes, a new tool to enhance your real Time QPCR applications."

Dr. Khalil ARAR, Director of Research & Development PROLIGO SAS

The advent of real-time PCR has substantially improved the quantitative accuracy and specificity of DNA sequence detection. An increasing numbers of technologies exists allowing the improvement of the performances of different parameters such as sensitivity, discrimination, throughput and multiplexing to name a few. However, not all target sequences are created equally. Numerous problematic sequence features and applications impact the ability to easily design probe specific to a particular target. For example, sequential “Gs” result in secondary structure that can block efficient hybridization, weaker bonds within “A-T” rich regions result in lower melting temperatures (Tm) that inhibit the use of efficient PCR conditions, and limited target regions can restrict the length of optimal probe and primer sequences impacting their functionality.
To overcome this, we developed a new generation of probes, called locked nucleic acids (LNA)-real time PCR probes. In this work, we describe the properties of this novel class of real time PCR probes. These probes containing LNA hybridize to complementary single stranded target DNA sequences with an increased affinity compared to oligonucleotide DNA probes. As a consequence of the incorporation of LNA residues into the oligonucleotide sequence, the melting temperature of the oligonucleotide increases considerably, thus allowing the design of short probes to be used as allele-specific tools in genotyping assays.

"Novel amplification and detection chemistries for real-time PCR."

Dr. Dirk Löffert, QIAGEN GmbH, Hilden

Real-time PCR has become a widely used technique for quantification of mRNA levels. Enzyme choice, reaction conditions, and detection methodology affect the specificity, sensitivity, efficiency, and ultimately the success of real-time PCR and RT-PCR assays. Discussion will focus on tools and techniques for avoiding gene expression artifacts and improving specificity and sensitivity of real-time experiments by novel reagent developments and primer-probe design.

"Highly sensitive analysis of allele-specific gene expression by MALDI-TOF MS."

Stephen Sharp, Sequenom, Inc., San Diego, Germany
Dr. Jurinke, Christian (cjurinke@sequenom.com)

Quantitative analysis of gene expression at the mRNA level is an important aspect of functional genomics. Here we present a novel method for quantitative gene expression analysis based on the MassARRAY system.

The protocol is a combination of competitive PCR and quantitative MALDI-TOF mass spectrometry. cDNA is mixed with a synthetic DNA molecule (the competitor or internal standard). The internal standard resembles the sequence of the targeted cDNA region in all positions but one single base. The method is highly sensitive (LOD 3 molecules), highly accurate (CV < 3%) and flexible in terms of assay design.

Data comparing the MassARRAY approach to other methods will be provided based on examples of analyzing p73 allele specific expression in different cell lines and analysis of paraffin embedded cancer tissue from clinical isolates.

Poster presentations

Poster Session: qPCR Application in Clinical Diagnostics

P1
Real time PCR method to differentiate between homozygous and hemizygous fish.

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The *AquAdvantage*™ gene construct opAFP-GHc2 contains "all-fish" transgene comprising the 5′-and 3′-regulatory sequences from an ocean pout (*Macrozoarces americanus*) Type III antifreeze protein (opAFP) gene and the growth hormone-coding sequence (GH) of the chinook salmon (*Oncorhynchus tschawytscha*). The *AquAdvantage* gene construct was injected into salmon eggs leading to the development of a fast growing *AquAdvantage*™ Salmon, The purpose of developing fast growing salmon was to improve the economics of aquaculture so that land-based systems become economically viable and at least as efficient as ocean-pen systems. This gene transfer results in two types of transgenic fish, homozygous and hemizygous. Currently the only way to differentiate between the two types of fish is traditional breeding mechanism, where a test cross is made before the actual one, to differentiate between homozygous and hemizygous fish. This method is very time consuming and results in high maintenance costs. To overcome this problem a rapid relative quantification real time PCR method has been developed using LightCycler® from Roche Applied Sciences. In this method quantification was performed in SYBR Green format. Suitable primers were selected from the Growth hormone coding region of the transgene, as well as from 18S housekeeping gene. A known sample from a homozygous fish was used as a calibrator. Our results suggest that this method is fast, highly reproducible and is able to differentiate between transgenic and a wild type fish. In addition it was able to detect homozygous fish with 88% accuracy. Currently we are working on developing a hybridisation probe format of the same method to improve the accuracy of quantification. A comparison of the data obtained from two different formats will be presented.

P2
Higher expression of RANKL in osteoporosis than in osteoarthrosis

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Receptor activator of NFkappaB ligand (RANKL) is a recently discovered key regulator of osteoclast formation and bone resorption. RANKL is expressed by osteoblasts/stromal cells and promotes osteoclast development and survival by binding to receptor activator NFkappaB (RANK), expressed on osteoclast precursors and mature osteoclasts. Deletion of RANKL gene in mice resulted in severe osteopetrosis and a complete lack of osteoclasts, while parenteral administration of RANKL resulted in severe osteoporosis.
The aim of the present study was to compare the expression of RANKL in bone tissue from patients with osteoporosis and osteoarthrosis and examine its association with bone mineral density (BMD).
Eighty-four female and male patients undergoing total hip arthroplasty due to osteoarthrosis (56 patients, aged 34-80 years) or osteoporotic femur fracture (28 patients, aged 53-90 years) were included in the study. Total RNA was isolated from the biopsies obtained during routine surgery for arthroplasty and the expression of RANKL was measured by real-time PCR.
Patients with osteoarthrosis had higher BMD of the femoral neck and hip than those with osteoporosis (P< 0.001). Consistent with this expression of RANKL was higher in patients with osteoporosis than in those with osteoarthrosis (P = 0.032). However, no association of RANKL expression with BMD of either the femoral neck or hip in the whole group of patients (P = 0.441 and P = 0.896 respectively) could be demonstrated.
Higher expression of RANKL in the bone tissue of patients with osteoporosis compared with those with osteoarthrosis implicates more intensive bone resorption in the osteoporotic patients. Lack of association with BMD could be the consequence of other factors, contributing to the regulation of bone remodelling, especially osteoprotegerin (OPG), which is the natural RANKL antagonist. It would be interesting to determine the expression of OPG and to examine the association of the RANKL/OPG ratio with BMD.

P3
Diagnostics of apple proliferation (AP) phytoplasma by real-time PCR

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Apple proliferation is a serious disease of apple spreading in many European apple growing areas. The causal agent of the disease is the apple proliferation (AP) phytoplasma that is thought to be transmitted by insect vectors. So far, there are no measures available to cure infected trees. Thus, the only possibility to control the disease is to prevent its spread by removing infection sources and planting disease-free material. However, such a strategy depends on efficient diagnostic tools that are highly sensitive and specific and that also have the potential for high-throughput testing. Here we describe the development of a novel approach for the diagnosis of AP phytoplasma that is based on a fluorogenic 5' nuclease PCR. In addition to targeting the pathogen's DNA, this approach also implements an internal positive control by simultaneously amplifying a gene of the host organism. Thus, uninfected plant material can be differentiated from false-negative results caused by PCR-inhibition. We demonstrate the high detection specificity of the novel approach for all AP phytoplasma strains by testing the method on apple trees infected with apple proliferation and on phytoplasma strains

causing diseases in other plant species. Unspecific amplification was neither observed with DNA of other fruit tree phytoplasmas nor with phytoplasma strains distantly related to apple proliferation. Moreover, compared to existing analytical diagnostic procedures for AP phytoplasma the new approach shows a higher detection sensitivity ranging over almost five orders of magnitude.

P4

A highly sensitive real-time PCR assay for the detection of HCV RNA in human cells

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In vitro cultures of human cell lines capable of supporting HCV propagation are critical for understanding viral infection and pathogenesis, as well as for evaluating anti-HCV compounds. We took the approach of infecting human hepatoma cell-lines with HCV sera derived from HCV patients. Currently, viral infection is monitored by measuring total cellular RNA followed by qualitative detection of HCV RNA via end point RT-PCR.
The need for specific detection of low abundance RNA and quantitative analysis directed us to develop a highly sensitive real time PCR assay specific for HCV. The assay is based on TaqMan method utilizing specific primers for the 5' untranslated region of HCV and a Fam-Tamra labeled probe, which covers most of the HCV genotypes. Reagents constitute the reaction mixture were optimized using cells spiked with HCV. For standardization of the extraction protocol and PCR amplification we included a house keeping gene analysis –human β-actin, which was performed alongside the HCV reaction. In order to determine absolute viral copy number, cells were spiked with a calculated HCV viral titer and a standard curve was generated for each experiment. The dynamic range of the optimized assay was between 10 - 10^5 viral RNA copies. This facilitates the detection of very few viral copies in cells infected with HCV sera relative to cells incubated with normal human sera.
This rapid and sensitive technique was utilized to analyze and compare infectivity of different human sera as well as to test activity of anti HCV compounds. Our experimental results demonstrate that real time PCR can serve as a convenient and powerful tool for determination of HCV RNA in cell lysates and for the evaluation of potential anti HCV agents.

P5

Sensitive and rapid quantitative detection of HCV RNA by real-time PCR

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The quantitation of hepatitis C virus (HCV) RNA sequences in plasma is used as a prognostic marker for individuals undergoing treatment with interferon and in the subsequent monitoring of their responses. Real-time PCR technology may provide more precise and sensitive method and affords a greater dynamic range than end-point detection methods.
We have developed a quantitative method for HCV RNA based on competitive reverse transcription polymerase chain reaction (RT-PCR) using the TaqMan technology and Rotor Gene 3000 (Corbett Research). A pair of primers and TaqMan probe were designed that are specific for the recognition of a highly conservative 5'-non-translated region in HCV genome. For accurate quantitation of the number of copies of HCV in samples containing unknown quantities we have used an internal control with a known concentration of RNA. The internal control is incorporated in the assay to monitor the extraction and amplification processes. For increasing the sensitivity and simplification of the test we have developed a single-tube reverse transcription PCR (RT-PCR) method using MLV-reverse transcriptase and "hot-start" Taq-polymerase.
HCV real-time RT-PCR test has a sensitivity of 100 RNA copies per reaction, with a dynamic range of detection between 500 and 10^7 RNA copies. In comparison with commercial PCR assay Amplicor HCV Monitor, the developed test is more sensitive and has a greater dynamic range of quantitation.
In conclusion, the high sensitivity, simplicity and reproducibility of the real-time HCV RNA quantitation, combined with its wide dynamic range, make this method especially suitable for monitoring of the viral load during therapy.

P6

Identification of topical anti-ageing compound using gene expression studies on Human reconstituted epidermis.

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Many clinical studies reported the positive effect of *all*-trans retinoic acid (RA) topical application on photodamaged skin and on wrinkles. In cell, retinoids bind to nuclear receptors and transactivate expression of genes containing specific response elements. In addition to these direct receptor-mediated transcriptional effects, retinoids are also known to have indirect activities leading to secondary responses and inducing gene expression. All these effects result in a complex pattern of transcriptional responses which are specific for retinoids.
In a previous study, we showed using cDNA macroarray, that a *Malva sylvestris* extract, studied in a reconstituted human epidermis model (RHE) , induces a gene expression profile very similar to that observed with retinoic acid. For quantifying and verifying the cDNA macroarray-identified differential expression of selected genes induced by the two different treatments, we developed real-time quantitative RT-PCR assays. We selected 3 genes regulated during epidermis differentiation and/or photoageing (involucrin, beta 1 integrin, SKALP) and 3 genes implicated on inflammation response (IL1 alpha, IL1 beta and IL8).
RT-PCR were performed in conditions to obtain a detection threshold of 10 copies and an efficacy up to 90% with standard curves and samples. Differentiated RHE were treated with the *Malva sylvestris* extract (3%) or with RA (1 M) for 24h. In order to limit inter-individual variability, we used a pool of treated RHE as samples.

We showed in the RHE model that the genes studied with these RT-PCR assays, were regulated in the same way with the two treatments confirming results obtained with cDNA macroarray method.
In conclusion, real time quantitative RT-PCR assays associated with macroarray method and reconstituted human epidermis model utilisation is a good strategy for identifying and studying new anti-ageing molecules.

P7

Development of a commercial available real-time PCR assay for the detection of Hepatitis-A virus: fields of application

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Presently commercially available PCR-based screening methods for quality control and safety of blood and blood products are hardly established. Here we describe the adaption and development of a CE-marked quantitative Hepatitis-A virus PCR assay. Hepatitis-A virus, the only representative of the genus Hepatovirus, is presently defined by only one serotype. Despite the limited heterogeneity of the amino acid sequence, a significant degree of nucleic acid variability has been observed among different isolates as well as within the itemized genotypes. In this work we studied a plasma donation, which contained HAV RNA but at the time of donation no HAV antibodies. It showed that quantification with our Real Art HAV LC PCR Kit by this donation was problematic. So it was necessary to develop a new, advanced HAV quantification Kit with increased sensitivity and specificity and kit characteristics in terms of sensitivity, specificity and performance will be discussed. The Hepatitis-A virus from the plasma donation was sequenced and fully characterized. Although most HAV isolates from Europe belong to genotype I this isolate seems to be a member of genotype III. This plasma donation demonstrates that a continuous observation of sequences and therefore a continuous improvement of commercial HAV PCR Kits is necessary, as HAV shows as much genetic heterogeneity among the strains as some other RNA viruses.

P8

A RQ-PCR APPROACH FOR QUANTITATION OF GLIVEC RESISTANCE ASSOCIATED MUTATIONS.

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Background: Glivec is a potent inhibitor of the constitutively active tyrosine kinase Bcr-Abl. Mutations in the Abl kinase domain were shown to abolish Glivec function and represent the main reason for resistance to Glivec. Early diagnosis and quantitation of such mutations could be important in order to tailor combination of Glivec with other Bcr-Abl targeting therapy.
Aim: We designed a mutation specific real time PCR method for detection of 5 different Glivec resistance associated mutations. The aim of this study is to assess specificity and sensitivity of the assay.
Methods: Real time PCR reactions using Taqman technology were constructed for mutations leading to the following amino acid substitutions: Gly250Glu, Tyr253His Glu255Lys, Thr315Ile, Met351Thr. Since the mutations are clustered, we could use three common forward primers and probes. Five different reverse primers were constructed as allele specific primers with their 3'-end complementary to the mutated nucleotide. They were tested for specificity using cDNA from healthy blood donors. PCR amplification before cyclus 40 was supposed to be unacceptable misannealing. In these cases incomplementary nucleotides were introduced in the ASO primers and annealing temperature was increased. Depending on how far from the 3'-end the incomplementary nucleotide was located and which nucleotide was choosen, we could vary the degree of destability of the primer. A couple of primers were designed for each mutation and the primers leading to optimal specificity and sensitivity were used in the assay.
PCR amplified abl kinase domain and templates produced by PCR mutagenesis were cloned into a Bluescript vector. A 10 fold dilution of 10E6 copies was amplified in the PCR assay to create standard curves, which could be used to quantify unknown samples and to assess the sensitivity of the assay in absolute copy number.
Results: Batches of absolute plasmid standards with known copy number could be produced for each targeted mutation. Repetitiv analysis of these plasmid standards were highly reproducible (p 0,0001 in a regression analysis). The degree of misannealing varied between the different PCR assays. Nevertheless misamplification could be minimized beyond cyclus 40 in every case. 10 copies of the plasmid standards generally gave amplification signals around cyclus 38 and at least 3 cycluses proximal to the misamplification signal, demonstrating that sensitivity could be set to 10 plasmid copies per ul. Sensitivity was also assessed using cDNA from a Gly250Glu positive patient sample diluted in Bcr-Abl positive samples not containing mutations. 1 ul mutation containing cDNA could be tracked diluted in 999 ul not mutation containing cDNA (Sensitivity 0,1%).
Conclusion: We describe a novel mutation specific RQ-PCR based metod for screening and quantifying Glivec-resistance associated mutations. As few as 10 copies can be detected in a background of 10 000 wild type genes.

P9

Identification and quantification of human polyomavirus BK (BKV) in renal allograft patients

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In the vast majority of humans, polyomaviruses are thought to persist in a lifelong latent state. However, in immunocompromised patients, reactivation of polyomavirus is an increasing problem. Renal-graft dysfunction due to infections of polyomaviruses of the BK type (BKV) has established a new diagnostic entity; BK nephropathy (BKN), or interstitial nephritis caused by BKV. It has become the most significant viral infection affecting renal allografts. Identification of BKV and monitoring of viral load during therapeutic attempts to treat BK nephropathy has proven to be a valuable adjunct tool for assessing therapeutic efficacy.

We have established a sensitive quantitative real-time PCR (Q-PCR) assay to quantify BKV in blood and urine. Samples from renal allograft recipients have been analysed on a routine basis over a time period of nearly three years. The National Hospital in Oslo, Norway, performs approximately 200 kidney transplantations annually. 159 patients have been tested for polyomavirus by Q-PCR in our laboratory. Among these, 34 were positive for BKV and 7 for the closely related polyomavirus strain, JC virus. 16 of the patients positive for BKV were positive in both urine and blood. Among these, 11 proved positive for BKV in concomitant biopsies, both immunohistochemically and by conventional PCR. As a consequence they were diagnosed having BK nephropathy.
Renal-allograft recipients with BKN are closely followed by monitoring of viral load (copies BKV/ml urine or plasma) by Q-PCR. Our real-time based approach has higher sensitivity compared to conventional PCR. In addition the TaqMan probe add increased specificity to the assay. However, deviations or variations within one log phase, are not considered clinical significant.
Conclusion: Quantitative real-time PCR has become an invaluable tool in evaluating the presence and role of polyomaviruses in nephritis aetiology. Identification and quantification of BK virus load in a renal-allograft recipient represent important steps in the clinical investigation potentially indicative for therapeutic response and prognosis.

P10
Multiplex real-time PCR assay for detection of HCV and HIV RNA and HBV DNA in blood donations

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Despite of donor serologic screening assays, there still remains a small but significant transfusion risk for each of the major viral agents (hepatitis B virus, hepatitis C virus and human immunodeficiency virus). The main risk is due to the failure of the screening tests to detect blood donations that are recently infected in the pre-seroconversion window phase of infection. In some countries molecular tests are used to detect viral genomes in blood donations, in particular HCV, HIV and HBV. Available assays do not allow screening huge amount of samples. The result is the necessity to pool plasma samples that considerably reduces sensitivity of assays.
We have developed simple, rapid, semiautomatic and maximum sensitive test that detect HIV, HCV and HBV in a single tube at the same time. The test is based on the technology of real-time PCR with TaqMan chemistry using Rotor Gene 3000 (Corbett Research). Particularity of the test is single-tube RT-PCR with MLV-reverse transcriptase and “hot-start” Taq-polymerase. The reaction buffer consists of 3 pairs of primers and 3 TaqMan probes. Each specific probe is labeled with a different fluorescent dye - Fam for detecting HCV, Joe for detecting HBV and Rox for detecting HIV.
For increasing the test sensitivity we have used the approach of ultracentrifugation of plasma samples. Two methods of DNA/RNA extraction were studied: manual and semiautomatic. We have used semiautomatic device KingFisher ml (Thermo Labsystems) for automation of extraction step and magnetic silica.
The sensitivity in the multiplex PCR assay was found to be 100 copies for HCV, 200 copies for HIV and 200 copies for HBV. The automation of the extraction and detection steps will allow analyzing up to 500 samples per day.
In conclusion, this TaqMan approach could be used as a single test to screen for HBV, HCV and HIV. Such an approach is a first step in Russia for the development of automation allowing a systematic screening of blood donations.

P11
Application of laser microdissection and real-time Q-PCR on immunocytochemically identified neurons of the human brain: results from post-mortem material.

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In order to understand the molecular mechanisms underlying human brain function in health and disease, it is crucial to study the expression of various mRNAs on postmortem brain. The brain is a complex tissue, which contains a heterogeneous population of cells with different morphological, neurochemical, functional and molecular characteristics. A brain homogenate will include information from all neuronal and non-neuronal cell populations making it difficult to make cell specific conclusions. Therefore, it is necessary to study the changes in expression profile at the single cell level in specific cell populations. The purpose of our study was to examine how different processing of the postmortem brain tissue could influence the availability of mRNA of immunocytochemically-identified neurons isolated by laser microdissection from human brain sections. We investigated the effect of fixation and immunocytochemical (ICC) procedure on RNA availability using real-time Q-PCR , without prior RNA amplification steps. Our material consisted of two different brain areas, hypothalamus and locus coeruleus, which were either paraffin-embedded or fresh frozen, from two control subjects with post mortem delay 4-7 hours. We examined two highly expressed in both areas genes -Elongation Factor 1a (EIF1a) and Cytochrome c oxidase (Cox 1)- as well as Arginine Vasopressin (AVP) and Tyrosine Hydroxylase (TH), the two latter are highly expressed in hypothalamus and locus coeruleus respectively. Both paraffin and frozen sections (6μm and 10μm thick respectively) of the same brain area were stained with a modification of the peroxidase-antiperoxidase ICC technique with reduced antibody incubation times. For all genes studied, the signal appeared in lower cycle threshold, range 2-5 cycles, when total RNA was extracted from frozen material, as compared with paraffin embedded material. The ICC procedure appears to slightly reduce the availability of mRNA. However, with any procedure used we were able to detect the genes of interest in each brain area examined. In conclusion, single neuron Q-PCR can be applied without prior amplification on postmortem human brain tissue using laser microdissection on immunocytochemically-identified sections.

Brain material was obtained from the Netherlands Brain Bank (coordinator R. Ravid)
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P12

Detection and quantitation of residual DNA in biopharmaceuticals by real – time PCR technique

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Although most biopharmaceuticals are highly purified, there is a theoretical concern that such recombinant products could be contaminated with oncogenic or bacterial DNA. A crucial part of the control of such biologicals is to ensure they do not contain more residual DNA than a safety limit suggested by the regulatory agency - currently the FDA has suggested a 100 pg per dose limit for residual DNA.
Confronted with increasing number of samples for surveillance it was essential to establish a high-throughput detection procedure to analyze the specimens. The current standard methods are time consuming, labor intensive, require large quantities of samples and they are even dangerous (e.g. radiation hybridization assay). Nucleic acid-based techniques, especially real-time PCR have the advantages of speed, specificity, and sensitivity for detection of bacterial host chromosomal DNA. In addition, real-time PCR has the ability to quantitate nucleic acid in real time. In this article we report about the development of the method for detection of residual DNA in recombinant pharmaceuticals using real-time PCR under universal conditions. We present two different types of detection and quantification of residual DNA namely by Taq-Man chemistry and by SybrGreen chemistry. A minimum detectable limit (MDL) of < 0.0033 pg DNA per ml in the protein – rich aqueous sample can be achieved.

P13

Characterization of mtDNA SNP typing using quantitative real-time PCR for forensic purposes with special emphasis on heteroplasmy detection and mixture ratio assessment

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The analysis of mitochondrial DNA (mtDNA) has proven to be highly useful in forensic casework, especially when degraded samples are analyzed which are not amenable to the typing of the highly informative nuclear short tandem repeat markers (STRs). However, unlike autosomal STRs, mtDNA testing does not provide definitive identification of individuals because all members of a matriline are expected to match each other due to maternal inheritance of the mitochondrial genome and the lack of recombination. Therefore, the principal limitation associated with forensic mtDNA typing is the low power of discrimination that is obtained when common mitochondrial types are present. Current mtDNA testing typically targets the variable base positions in the non-coding control region of the mitochondrial genome by sequencing both strands, and most laboratories restrict their investigations to one or two hypervariable regions (HV1 and HV2), comprising approximately 600 base pairs (~3.6 % of the entire mitochondrial genome). In the last couple of years it has become increasingly recognized that assays targeting single nucleotide polymorphisms (SNPs) are well suited for efforts to gain additional information in mtDNA testing, as the majority of the variable sites show only two alleles. Because many informative SNP sites reside in the coding region of the mitochondrial genome SNP typing assays are superior to sequencing due to their ability to address particular sites selectively. This circumvents the potentially serious problem of obtaining genetic information that correlates to a phenotype (e.g. disease). We investigated the forensic applicability of real-time detection PCR using TaqMan probes targeted to the highly discriminatory mitochondrial control region SNP 16519 T/C for several reasons: 1) it's large linear dynamic range in terms of target-molecule input number allows – along with the short amplicons that are obtained - the analysis of a broad spectrum of samples differing in DNA quantity and quality with a single protocol, 2) the homogeneous format of the assay avoids potential cross-contamination of samples with PCR products and makes it easy to automatize, 3) the quantitative information that can be obtained allows the formulation of objectively-based criteria for distinguishing between authentic signal and contamination and 4) the multicolor capability of real-time PCR instruments enables the simultaneous interrogation of both base-states of the SNP under investigation by means of differentially labeled allele-specific hybridization probes. The last point is particularly important because of the potential of mtDNA to manifest heteroplasmic mixtures on a continuous scale. The results of a population study on 135 paternity trios with known control region sequences showed that 16519 can be reliably typed with the TaqMan approach without a need to run samples in replicates. For both variants the linear dynamic range was at least 5 orders of magnitude with a lower end sensitivity of approximately 10 target molecules and an apparent single cycle PCR efficiency during the exponential phase close to 100% for complex genomic DNA as well as for non-linearized plasmids used as template. Defined mixtures of plasmids containing 16519T and C could be detected and quantitated reliably down to the 5% level for either variant with a lower end sensitivity of a total of 200 target molecules. Finally, the estimated mixture ratios for three heteroplasmic and two homoplasmic paternity DNA samples were in excellent agreement with the results obtained by the typing of approximately 300 to 400 clones per sample containing the entire PCR amplified mitochondrial control regions.

P14

QUANTITATIVE REAL-TIME RT-PCR ANALYSIS OF PSA AND HK2 MRNA IN CIRCULATIG CELLS FROM PATIENTS WITH PROSTATIC DISORDER

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Prostate cancer (PCa) is the second most common cause of male cancer death after lung cancer. Although immunoassays of prostate specific antigen (PSA) are routinely used for early detection of PCa, there is a widely acknowledged need for more reliable identification of PCa both from benign prostatic hyperplasia (BPH) and from PCa lesions likely to remain indolent. QRT-PCR analysis of circulating nucleated blood cells may offer a useful tool for finding cases of clinically significant PCa. We here report internally standardized real-time RT-PCR assays for PSA and hK2 mRNA in circulating cells.

In the RT-PCR reaction, a terbium chelate-labelled specific probe and a complementary, QSY-7-labelled quenching probe, were used. The terbium-label is environment sensitive; its fluorescence is lower when coupled to single stranded DNA than when free in a solution. When the probe anneals to a complementary target amplicon the 5'-3' exonuclease activity of the polymerase detaches the label from the probe. This results in an enhanced long lifetime terbium fluorescence signal. The signal to noise ratio is further increased through the use of separate quencher probe that anneals to intact lanthanide probe molecules.
The variations of cDNA synthesis and DNA amplification efficiencies were corrected by using an exogenous PSA-like internal standard mRNA (mmPSA). This enables absolute quantification of the target transcripts. The detection limit for both assays is at best 50 mRNA molecules per 1µg total RNA compared to standard PSA and hK2 RNA.

P15
Rapid and highly sensitive pharmacogenetic diagnostics using real-time PCR

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Adverse drug reaction can be frequently observed during treatment of several diseases and may occur due to genetic reasons. According to a meta-analysis published by Lazarou et al. (1998) drug-induced site reactions rank on place four of death rate. However, it is not known which of these cases of death have a genetic cause. A huge number of single nucleotide polymorphisms have been described in the last ten years. Many of these SNP's were identified within drug receptor, phase I and phase II genes. They may alter enzyme activity and thereby have influence on the individual drug metabolism. It has been described that drug treatment of patients can lead to severe adverse drug reactions including death due to enzyme deficiency from genetic alterations.
In the last decade the direct detection of single nucleotide polymorphism using the versatile polymerase chain reaction or PCR-related methods has revolutionized the field of medical diagnostics. Nevertheless, conventional PCR-based methods require a post-reaction analysis by gel electrophoresis, which is time-consuming and prone to cross-contamination. Also, carrier of a single nucleotide polymorphism need to be identified by further steps, for example RFLP.
A highly specific detection system is required to identify doubtless heterozygous and homozygous carrier of a genetic polymorphism. The artus GmbH is specialised in the field of real-time PCR and offers pharmacogenetic assays for use with the LightCycler® instrument (Roche Diagnostics), which allows genotyping of four important genes. The exon 14 skipping within the dehydropyrimidine dehydrogenase gene (DPD), three SNP's of thiopurine S-methyltransferase (TPMT), two SNP's of methylenetetrahydrofolate reductase (MTHFR) and seven of N-acetyltransferase 2 (NAT2) can be analysed. Using these assays the number of adverse drug reactions due to genetic reasons can be reduced, when substrates like 5-fluorouracil, thiopurines, methotrexate or isoniazid are administered. In addition, genotyping of these genes can help to optimise drug dosage and duration before starting patients therapy. All of these assays have been developed according to the requirements of the technical documentation and are CE-certified.

P16
Biological reference materials for *BCR-ABL* RNA detection by RQ-PCR assays for therapeutic monitoring and optimization

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Imatinib is a forerunner of molecularly targeted treatment approaches for leukaemia, demanding reliable detection of the *BCR-ABL* fusion and quantitative RT-PCR to monitor therapeutic response. Basing treatment decisions upon molecular monitoring for minimal residual disease (MRD) is currently in its infancy and there is an urgent need for standardization of methodology, facilitating comparison of data from international trial groups. A further key step towards achieving this goal is the establishment of biological standards for leukemia associated fusion genes. We started a collaborative project with the NIBSC in order to develop standards for real-time PCR to detect *BCR-ABL* transcript levels using lyophilized cells. Test materials were then evaluated in 22 laboratories spread across 15 countries. K562 cells were lyophilized at a concentration of 3×10^6 cells/ml. Vials were stored at 20°C and dispatched at room temperature. Gene sets obtained from plasmids containing inserted cDNAs corresponding to control (*ABL*) and fusion M *BCR* (*BCR-ABL*) genes were sent to each laboratory (kindly provided by Ipsogen, France) together with four vials of cells. Participants were requested to perform four independent assays and the cell samples were to be tested at neat, 10^{-1}, 10^{-2} and 10^{-5} dilutions. The majority of laboratories in this study used the EAC standardized RQ-PCR protocol and primer and probes for the two genes. Preliminary statistical analysis was done on the ratio of copy numbers of *BCR-ABL*:*ABL*, expressed as a percentage. Accelerated degradation studies were also done in order to determine the stability of the reference material. The estimated ratios of *BCR-ABL*:*BCR* were reproducible across the majority of laboratories with a mean of 100% (50-200%) excluding the results from two laboratories. Despite distribution of QC materials at ambient temperature, the lyophilized K562 cells consistently yielded very high quality RNA, with control gene (*ABL*) and *BCR-ABL* expression levels being comparable to those observed in primary patient material. Preliminary RQ PCR results in the accelerated degradation studies showed little change in the RNA titers of samples incubated at 6 months at 37°C for 6 months or at 45°C for 2 months indicating that the lyophilized reference material is very stable. Our preliminary results indicate that lyophilized K562 cells can be used as a reference material for RQ PCR assays. This study open the way to establishing reference materials for leukemia associated fusion genes, which is critical for the reliable use of MRD monitoring based on abnormal gene expression such as fusion transcripts for therapeutic decision making.
We would like to proposal our material to the WHO as an international reference material. Actually, our data suggest that freeze dried cells are suitable for reference material of the dosage by RQ-PCR of any transcript in the area of biology.

P17

Real-time quantification of gene transcription in the causative agents of Dutch-elm disease

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Phytopathogenic fungi produce a spectrum of cell wall degrading enzymes among which pectinases are probably the most important since they depolymerize pectin, one of the principal components of the plant cell wall and middle lamella. One of these pectin-degrading enzymes is polygalacturonase (PG). PG hydrolyses glycosidic linkages between galacturonic acid residues in polygalacturonan, a major fraction of plant pectin. As PG plays a vital role in the invasion and colonization of host plants by pathogenic fungi, it is important to evaluate the *pg* gene expression in fungal cells recovered from different developmental stages of aggressive (virulent) and non-aggressive (less virulent) strains. Differential gene expression analysis in biological research is increasingly facilitated by the use of real-time reverse transcription PCR (RT-PCR) as the most suitable method for the detection and quantification of mRNA. It offers high sensitivity, good reproducibility and a wide quantification range. Here we describe the use of real-time RT-PCR analysis in the study of *pg* mRNA abundance in yeast-like and mycelial cells of the Dutch-elm disease pathogens *Ophiostoma ulmi* (H5, non-aggressive strain) and *Ophiostoma novo ulmi* (H327 and MH75, aggressive strains). Our initial results indicate that comparable levels of *pg* transcripts were measured in H327 and MH75 while the level in H5 was about 2 times lower. It was also observed that mycelium had 1.5 times more abundance of *pg* mRNA as compared to yeast. We discuss the implications of the results to the pathology of Dutch-elm disease.

P18

Evaluation of a real-time PCR for the diagnosis of adenoviral keratoconjunctivitis in clinical eye-swabs

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Human adenoviruses (HadV) are the cause of epidemic keratoconjunctivitis (EKC). Because HadV are highly contagious outbreaks of EKC in hospitals and community institutions present an important public health problem. During a nosocomial outbreak rapid laboratory diagnosis is required to confirm the clinical diagnosis and to adopt appropriate measures. Nested PCR has been shown to be the most sensitive method to detect HadV in clinical samples. A lightcycler real-time PCR-assay using the consensus primers and the dual labelled taqman-probe described by Heim et al. (J Med Virol 70:228-239, 2003) was optimized for the use with the Qiagen QuantiTect™ Probe PCR Mastermix. To evaluate the diagnostic power of the assay for the diagnosis of EKC, stored extracted DNA of 209 eye swabs which had been collected from 1999-2003 was tested. The eye swabs were routine samples from the ophtalmologic departement of the university hospital of Würzburg with the clinical suspicion of EKC. 170 had been shown to be positive for HadV by conventional nested PCR. In addition 39 negative samples were randomly selected. The real-time PCR detected adenoviral DNA in 156 of the positive samples (91,8%). All 39 negative samples tested negative (100%). Because the stored DNA was exposed to repeated freeze and thaw cycles, the 15 samples with discordant results were extracted again from original sample material stored at -20ºC and tested with both PCR methods. The newly extracted DNA showed concordant results in all 15 cases. 6 samples were positive, 9 samples were negative. Quantifying viral loads of swabs is not reasonable due to the heterogeneity of such samples. Nevertheless absolute quantification of the adenoviral DNA using an external standard curve showed that many of the samples contained immense amounts of viral DNA (> 10^7 copies/ reaction). This is probably one of the reasons for the high contagiousness of adenoviral EKC. The evaluated real-time PCR-assay is a sensitive and specific tool for the diagnosis of EKC caused by HadV. Compared to the conventional nested PCR the real-time assay is much faster, easier to handle and avoids carry-over contamination due to the signal detection in sealed reaction vessels.

P19

Real time quantitative PCR machines in onco-hematology: a cost effectiveness analysis

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During program EAC (Europe Against Cancer), a standardized protocol of Real Time Quantitative PCR (RQ-PCR) in onco-hematology was developed by using fluorescent probes (TaqMan chemistry) on the ABI 7700 (Applied Byosystem). Since, several companies propose apparatuses allowing the use of such technologies. However no study ensure that results were identical when using different machines and no information on the relative cost of the results in this precise context is available. So we answered these questions with a cost-effectiveness approach.
By using the primers and probes defined by the EAC, we measured the expression of 3 control genes or CG (*ABL*, *GUS* and *TBP*) and 3 fusion transcripts or FT (m-*BCR/ABL*, M-*BCR/ABL* and *AML1/ETO*). This study was carried out on dilutions of RNAs from cell lines and the quantification was done by using a range of plasmides. The same sample is systematically analyzed in parallel on the evaluated machine and the ABI 7700 considered as the machine of reference. For each machine, the prices of the apparatus and correspondent consumable were raised as well as time necessary to the execution of an experiment.
Six machines were evaluated successively within the framework of this study. The assays enable reliable quantification on all the instruments, producing calibration curves with strong correlation and presenting the same sensivity on 10 copies of molecules. Moreover, the reproducibility and repeatability analysis showed low intra-assay and intera-assay variations. Furthermore, beside this the organisation of a workshop allowed the multicentric comparison of RQ-PCR analysis. The results for this

multicentric analysis are excellent taking into account the inexperience of operators. Because the effectiveness criterion is not significantly different from one machine to another, a costing of the analysis by patient enabled us to carry out a classification of the machines. Also we showed that the choice of the reagents accentuated the differences in cost between the machines. Lastly, we built a performance index (cost function) which depends on number of patient samples in the laboratory and then we analyze the variations in the classification.
Here we show that the primers and probes defined during program EAC, in the context of this evaluation of the residual disease in onco-hematology, are usable on all the machines of RQ-PCR tested. Moreover, the protocol developped by the EAC does not require any adaptation, and that affects neither the results, nor the sensitivity of the technique. The cost assessment then give an economic information to choose a strategy. We suggest that our study may serve as an example for help to decision making concerning the modalities of choise of seach innovative technology in the biologicl area.

P20

Quantitative real-time PCR using sequence-specific primers to discriminate between donor and recipient specific mitochondrial DNA polymorphisms

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Background: Mitochondrial DNA (mtDNA) contains genetic information specific for each individual. MtDNA polymorphisms can be used to evaluate (micro-) chimerism after transplantation and survival of blood platelets after transfusion. We developed sequence-specific primers that enabled us to detect single nucleotide polymorphisms in the three hypervariable regions of the D-loop of human mtDNA. Quantification of the amount of mtDNA is important for use of this method in a clinical setting.
Methods: mtDNA was extracted from whole blood and buffy coats (Qiagen/Gentra extraction). Total DNA concentration was determined by measuring the A260/A280 ratio and was set to 30 ng/µl. Primers were designed with the Primer3 program. Quantitative real-time PCR (qPCR) was performed with the sequence-specific primers using SYBR Green (ABI supermix). Tenfold dilution series (1-10^8) were used to determine the sensitivity of the qPCR method.
Results: Using sequence-specific primers and SYBR Green we were able to discriminate between mtDNA containing and mtDNA lacking certain single nucleotide polymorphisms. Incorporating extra mismatches in the specific primers resulted in most cases in an improved discriminating power. The dilution series revealed a high sensitivity with a dynamic range of 10^6.
Conclusions: The use of sequence-specific primers for the discrimination and quantification of mtDNA derived from different human cells is a promising method to identify donor and recipient cells.
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P21

A two-reaction real-time PCR assay covering the entire spectrum of human adenoviruses for early identification of patients at high risk of disseminated disease

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Adenovirus (AdV) infection in the course of allogeneic stem cell transplantation (SCT) is associated with high transplant related morbidity and mortality. Disseminated AdV disease is lethal in most instances. Early detection of AdV infection and identification of patients carrying a high risk of disseminated disease therefore remain a major challenge. Yet, many of the commonly used diagnostic approaches based on PCR analysis do not effectively cover all potentially relevant AdV species. In a recently published paper we presented a five-reaction RQ-PCR assay for reliable diagnosis of invasive AdV infection prior to clinical signs of virus disease (*Lion et al. Blood 102(3):1114-1120; 2003*). Using this approach, we observed in a number of instances that AdV detection in stool preceded the onset of AdV viremia, thus raising the possibility that intestinal infections represent a potential source of virus dissemination in a subset of patients. To address this question, we have monitored 80 consecutive pediatric patients transplanted at our center for the presence and the load of AdV in stool and in PB. In order to substantially reduce time and costs for AdV screening we have established a two-reaction RQ-PCR assay permitting sensitive detection and quantification of all 51 currently known human AdV serotypes. The specificity and sensitivity of the novel two-reaction assay is comparable to the five-reaction RQ-PCR assay. Twenty two (28%) patients tested positive in serial stool samples, revealing adenoviruses of the subgenera A, B, C, D and F, with strong predominance of subgenus C. Fourteen patients revealed only low levels of AdV positivity in stool, not exceeding 10E5 copies/g. None of these patients have shown viremia during the posttransplant course. Eight patients had peak AdV levels ranging from 10E5-10E11 copies/g of stool. In six of these eight patients we were able to detect rapid virus proliferation in the intestinal tract to an extremely high AdV load. In four cases these kinetics preceded dissemination of the virus by several weeks.
Our observations may therefore indicate a particularly high risk of progression of intestinal to invasive AdV infection in patients with rapid virus proliferation in the intestinal tract. In these patients the monitoring of AdV in stool may therefore permit timely onset of appropriate antiviral treatment, in attempts to prevent disseminated disease

P22

Development and analysis of a multiplex RT-PCR assay for combined expression screening of melanoma drug resistance target genes

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During the last decade, microarray analysis led to the identification of a large number of genes that are differentially expressed in disease processes. After validation, identified target genes can be either used for functional assays resulting in putative drug targets or for the screening of different samples (e.g. biopsies, cell lines, blood) in order to identify molecular markers for diagnostics.
For a fast and reliable screening of multiple putative diagnostic markers involved in melanoma drug resistance, we developed an assay for a semi-quantitative expression analysis of 11 drug resistance target genes recently identified in a gene expression profiling study. A multiplex RT-PCR (mRT-PCR) assay was developed allowing the gene expression screening of 11 target genes and 1 housekeeping gene in one reaction. Combining mRT-PCR with microfluidic fragment analysis, we were able to resolve, size and quantify 12 mRT-PCR products ranging from 116–582 bp. To validate the quantitative performance of our assay, the determined expression ratios were compared with data from microarray- and Northern-blot analyses. 8 out of 11 target genes were reproducible in terms of expression ratios, indicating these genes as informative for screening approaches. Applied to 5 melanoma cell lines, the mRT-PCR assay clearly identified differential expression of 6 target genes.
In conjunction with recent data, the results point to the usability of APOD, CYR61 and IL1B as marker genes for etoposide resistance in melanoma cell lines.

P23
Systematic Multiplex PCR and RT-PCR analyses of changes in copy number and expression of proto-oncogenes and tumor suppressor genes in cancer tissues and cell lines

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Systematic Multiplex RT-PCR (SM RT-PCR) is distinguishable from other multiplex RT-PCR methods by utilization of primers that amplify sequences that fall within a single exon of the genes. Gene expression is measured semi-quantitatively, using genomic DNA that carries equivalent copy numbers of individual genes as a calibration standard. We previously developed experimental systems to study the expression of 68 human glycosyltransferase genes and 39 human Hox genes. We analyzed the expression of those genes in human adult tissues and obtained more informative data on the expression of glycosyltransferase genes and Hox genes in human adult tissues than any other paper published. Furthermore, we surpassed in quantity all the information combined from previous publications. The panoramic view of a total of 1836 (68x27) and 1014 (39x26) expression data demonstrated that some of those genes were differentially expressed whereas others were ubiquitously expressed.
Here we report the establishment of a mini SM RT-PCR system of proto-oncogenes and tumor suppressor genes and the expression analysis of those genes in human adult tissues. The results show that some genes are expressed in a tissue-specific manner. Because of the crucial role that those genes may play in carcinogenesis, we have also examined gene expression in cancer tissues and cell lines using this system. BRCA1 and CDKN2A showed enhanced expression in cancer tissues and cell lines whereas NRAS and BRCA2 showed enhanced expression in cancer cell lines. KIT1 exhibited decreased expression in cancer tissues and cell lines, and FOS1 and NF1 showed decreased expression in cell lines. Because the percentages of cells multiplying vary among normal and tumor tissues and cell lines, the results may be indicative of their role in cell proliferation, rather than their role in carcinogenesis. In addition to the expression analysis, we present our demonstration that the SM RT-PCR system, which was developed for the cDNA expression analysis, could also be used successfully for more exquisite analysis of copy number changes in genomic DNA. We observed an increase in band intensity in NRAS gene in MCF7 and BT20 breast carcinoma cell lines and a decrease in HRAS, TP73, CDKN2A, and CDKN2B genes in most of the breast and prostate cell lines examined. The increase in copy number in the NRAS proto-oncogene in cancer cell lines is logical considering the gene's role as an accelerator in carcinogenesis. The corresponding logic applies to a decrease in copy number of TP73, CDKN2A, and CDKN2B tumor suppressor genes that act as brakes. However, the decrease in copy number of HRAS proto-oncogene was unexpected, which leads us to suspect the presence of tumor suppressor genes in the vicinity of this gene on chromosome 11p15.5.

P24
Simultaneous determination of fetal trisomies 18 and 21 by real-time quantitative PCR

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The detection of gross chromosomal abnormalities is a major focus of prenatal diagnostics, of which the most common cytogenetic anomaly in live births is trisomy 21, also known as Down's Syndrome. Currently, prenatal diagnosis of genetic anomalies relies on invasive procedures such as amniocentesis and chorionic villus sampling (CVS), from which the full fetal karyotype is usually determined using cultured cells. The two week of cultivation and subsequent analysis has proven to be associated with considerable parental anxiety. This delay is also associated with considerable medical problems in those situations requiring therapeutic intervention. In order to address these needs, more rapid methods for the prenatal diagnosis of fetal chromosomal aneuploidies have recently been developed and implemented, such as multi-color fluorescence in situ hybridization (FISH) as well as quantitative fluorescent PCR (QF-PCR) analysis of short tandem repeats (STRs). With the advent of real-time PCR it is now possible to measure concentrations of nucleic acid sequences with an accuracy that was not deemed possible only a few years ago. Examples are the analysis of gene expression and gene duplications / losses, where two-fold differences in nucleic acid concentration are routinely determined.
We have investigated whether real-time quantitative PCR (qPCR) could be used for the diagnosis of chromosomal anomalies, in particular the aneuploidies such as trisomy 18 and 21, where the difference in copy number is only 50 %. This approach was first tested in a pilot study, wherein we were able to detect cultured trisomy 21 samples with 100 % specificity and sensitivity. We have now modified this test to permit the simultaneous analysis of trisomies 18

and 21 and have demonstrated that our approach can be used for the highly reproducible and robust detection of only 1.5 fold differences in gene copy number. Our studies have also underscored that several criteria need to be met concerning template preparation and primer purity.

P25
Identification and quantitative measurement of BCR/ABL transcripts using quantitative real-time PCR (Q-PCR)

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The BCR/ABL translocation genotype, also known as Philadelphia chromosome, is present in 99% of patients diagnosed with chronic myelogenous leukaemia (CML)[1]. The reverse transcriptase-polymerase chain reaction (RT-PCR) has become widely adapted and used for monitoring minimal residual disease (MRD) after allogeneic stem cell transplantation in CML patients. Our lab performs analysis of approximately 225 samples a year. In average these samples represent approximately 110 follow-up patients and 45 newly diagnosed patients.
Recently, diagnostics have been performed using qualitative RT-PCR. We have newly established quantitative real-time PCR as a tool in the detection and quantification of the BCR/ABL fusion transcripts in our lab. Parallel analysis of quantitative real-time PCR and the conventional qualitative RT-PCR was performed on 20 samples from different patients. A good compliance was documented between the two methods. Quantitative real-time PCR provides relative quantification of BCR/ABL fusion transcripts compared with total amount of ABL transcripts. Clinicians are provided with a more sensitive and reproducible method for monitoring MRD. Also, quantitative real-time PCR is the less time-consuming of the two methods.
Martinelli et al, Haematologica, 1998. ref 7,8,11

P26
Optimised amplification of multicopy Y-chromosome sequences for the improved quantitative measurement of fetal male DNA in plasma by real-time PCR

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The quantitative measurement of fetal DNA levels in maternal plasma has opened a new approach for the noninvasive prenatal diagnosis. One of the main applications is the prediction of fetal sex by the real-time quantitative PCR technique, as in the assessment of X-linked disorders. The amplification of Y-chromosome specific sequences is also the main research tool applied for the determination of fetal DNA levels in correlation with pregnancy related disorders, including common trisomies, preterm labor and preeclampsia. Even though it is only applicable to 50% of the samples due to it's restriction to male pregnancies, this procedure is very straightforward and simple to perform. The precision and reproducibility of the quantitative measurements can greatly be improved by usage of Y-specific sequences that are present in more than one copy per Y-chromosome. The possibility to use of an increased number of amplification targets per Genome Equivalent has been identified for a long time, but it has not been widely exploited. We amended an existing protocol specific to the multicopy locus DYS14 and evaluated it's performance in comparison to the currently used SRY targeting protocol. Our evaluation shows that the altered protocol performs with an increased precision and sensitivity. Furthermore we present possible approaches for the standardized scoring of low template amplification to exclude false positive results.
In conclusion, the application of real-time quantitative PCR for the measurement of multicopy Y-specific sequences is with it's reduced variability an optimal tool for the assessment of fetal DNA concentration in maternal plasma and for the investigation of elevated levels under a variety of pregnancy related pathologies.

P137
Exogenous and endogenous controls for qualitative and quantitative detection of seminal cell-associated porcine reproductive and respiratory syndrome virus (PRRSV) RNA

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Endogenous and exogenous control assays for the qualitative and quantitative detection of relative PRRSV RNA copies in the cellular fraction of semen were developed. For reference and virus RNAs one-step real-time reverse transcription polymerase chain reaction (RT-PCR) assays on the basis of hydrolysis (TaqMan) probes were optimized to yield optimal amplification efficiency. The *UBE2D2* (*UBCH5B*) mRNA coding for the ubiquitin-conjugating enzyme E2D 2 was selected as the endogenous control. Due to its low expression in seminal RNAs it represents an appropriate control for PRRSV molecular diagnostics, especially for the analysis of persistent infections associated with low copy numbers of viral RNA. Since porcine semen expressed the *UBE2D2* mRNA at a variable (up to 45-fold differences in expression), but detectable extent in all samples analysed (n = 28), this endogenous control can be used for qualitative detection of PRRSV RNA. The synthetic exogenous reference (exogenous spike) – a non-metazoan RNA – is based on the photosynthesis gene ribulose-1,5-bisphosphate carboxylase/ oxygenase large subunit from *Arabidopsis thaliana*. In contrast to the endogenous control, the exogenous spike can be used for quantitative analysis of viral RNA if spiked to the seminal cell pellet at an exact copy number. Principally, both reference RNAs can control for transport conditions, RNA isolation performance and amplification efficiency.

Poster Session: qPCR Application in Microbiology & Virology

P27
Development of real – time PCR assay for *Chrysanthemum stem necrosis virus*

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Chrysanthemum stem necrosis virus (CSNV) was first reported in Brazil in 1995 on *Chrysanthemum* (Alexandre et al., 1996). It was also found on tomato and in mixed infections on ornamental plants: *Eustoma grandifolium, Callistephus* sp., and *Senecio x cruenthus* (Alexandre et al., 1999). In Europe only a few reports from UK and Netherlands on *Chrysanthemum* were made until now (Verhoeven et al., 1996, PH Newsletter, 2003). Survey of Tospoviruses in Slovenia, revealed CSNV in ornamental plants *Chrysanthemum* and *Gerbera*. In order to distinguish CSNV from other Tospoviruses specific primers for CSNV were developed and used in RT-PCR assay (Ravnikar et al., 2003). We further developed diagnostic assay by constructing primers and MGB-probe specific to CSNV using Primer Express® software. Internal control COX was included in assay in order to control the RNA extraction procedure. Reverse transcription step was performed separately; products of this reaction were later used in real-time PCR reaction. Results of testing the specificity and cross-reactivity of CSNV specific primers and probe on different CSNV isolates, closely related Tospoviruses, and various host plants will be presented. Advantages of molecular diagnostic methods in laboratory testing of CSNV will be discussed.

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P28
Automated system for isolation of bacterial DNA

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Over the last decade, clinical research has become increasingly dependent on nucleic acid based methods. A prerequisite for these methods is access to high quality DNA and/or RNA. We have previously introduced systems for automated isolation of DNA and RNA from human blood, cells and tissue samples. Based on two of our automation platforms, EZ1 and BioRobot M48, we have now developed methods for the isolation of DNA from bacteria. The methods are based on proven MagAttract chemistry, which exhibit known QIAGEN quality.
Until recently, the gold standard for identifying most bacteria species has been the culture method. This method is time consuming and laborious and is unfit for routine screening. The introduction of PCR and real-time PCR has greatly improved both efficiency and specificity for detection. However, several factors impede the optimal use of the high sensitivity of these methods. In particular, all sensitive and specific PCR assays require nucleic acids free from inhibitory substances and with high reproducibility. In addition, these methods are especially vulnerable to cross-contamination. Our results show that both EZ1 and Biorobot M48 is time saving automated systems which gives high-performance DNA well suited for sensitive PCR assays and without the risk of cross-contamination.

P29
Preliminary results 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. The virus has a quarantine status in European Union and in Slovenia. Except from *Prunus* species, numerous other woody as well as herbaceous species are known to be PPV hosts. However, the PPV host range can not be considered as final since new host species are continuously detected. PPV is transmitted by insects in a non-persistent manner. Typical symptoms of sharka were first observed in Slovenia in 1987 in several apricot, peach and plum orchards. Since 1988 DAS-ELISA method was used for testing. PPV was detected in intensive orchards in nurseries and on single trees throughout Slovenia on plums, peaches and apricots. Since 2001 IC RT-PCR is used for testing important samples or to confirm the ELISA results. The virus is irregularly distributed in the plant therefore successful detection of PPV depends on sampling, the organ sampled, the time of the year and sensitivity of the test method. At high temperatures the virus concentration in plants decreases and detection is less reliable. Real-time PCR assay for detection of PPV was developed. Primer Express software was used to design primers and minor groove binding probe (MGB). The conventional TaqMan probe could not be designed due to multiple mismatches in target PPV sequences from GenBank. Concentrations of primers and probe were adjusted for optimal performance of the assay. Serial dilutions of total RNA were used to compare the sensitivity of conventional and real-time PCR. To facilitate the work in the laboratory, reverse transcription was performed on RNA prepared by immuno-capture or on isolated total RNA.

P30

Nucleic acid sequence based amplification (NASBA) *Chlamydia trachomatis* 16S-rRNA with Real-time detection

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Chlamydia trachomatis is one of the most widespread sexually transmitted infectious pathogen in the world. Being mainly asymptomatic chlamydial infection has a great risk of development PID and other complications. High sensitive and specific molecular diagnostic assays are now considered to be preferable for screening this infection. Among different amplification technologies NASBA has several advantages being an effective tool for RNA isothermal amplification with reduced risk of contamination. Development technology of Real-time amplification detection with fluorescent-labeled probes make NASBA widely available and suitable for routine use.
We have developed primers and Molecular Beacon (FAM-BHQ1) specific for 16S-rRNA of C.trachomatis. Nucleic Acids (NA) were extracted from McCoy cell culture infected with Chlamydia trachomatis by the NucliSence® Isolation Reagents according the original protocol. Ten fold dilutions of NA were prepared. NASBA amplification and Real-time detection with developed primers-probe set and NucliSens® Basic Kit was carried out on NucliSens EasyQ Analyser (bioMerieux, the Netherlands). From the same samples dilutions an aliquots were used for PCR amplification and electrophoresis detection with primers for fragment of C.trachomatis cryptic plasmid. Detection limit of NASBA Real-time was at least one order of magnitude greater then PCR. Because chlamydial cell contain 16S ribosomal RNA much more then mulicopy plasmids, so the efficiency of RNA-amplification seems to be greater in comparison with DNA amplification.
NucleSens® Basic Kit – based reagents with the designed MB-probe for Real-time detection of 16S rRNA C.trachomatis could be used as ultrasensitive assay for screening C.trachomatis in clinical samples.

P31

Detection of *Mycobacterium avium* subsp. *paratuberculosis* in bovine semen by real-time PCR

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Paratuberculosis, a chronic enteric disease in ruminants also known as Johne's disease, is caused by *Mycobacterium avium* subsp. *paratuberculosis* (*M. ptb*). Control of the disease is complicated by the facts that clinical symptoms develop at a very late stage and bacteriological cultures may need up to 16 weeks to form visible colonies. The rapidity, sensitivity and specificity of real-time PCR would greatly benefit the detection of *M. ptb*. Although semen is not a common way for spread of paratuberculosis, isolation of *M. ptb* from semen has been reported. Therefore, there is a demand for testing semen intended for use in paratuberculosis free herds. We have applied the following method on artificially infected bovine semen. Samples of diluted *M. ptb* free semen were spiked with *M. ptb*, counted in bürker-chamber since CFU-count usually underestimates the number of detectable genomes. The *M. ptb* suspension used was also washed from free DNA to avoid overestimating the sensitivity of the test. Incubation with lysisbuffer and proteinase K followed by mechanical disruption by beadbeating released the DNA, recovered with phenol/chloroform extraction. Real-time PCR with a fluorescent probe targeting the insertion element IS900 could detect as few as 10 *M. ptb* per sample of 100 µl semen. PCR-inhibition could be monitored by the addition of a mimic molecule to the samples and it was shown that the applied method was sufficient for removing inhibiting components. Further evaluation on clinical samples will be done.

P32

A Validated *In-House* PCR Method for the Detection of *Chlamydia trachomatis* from Urine and Swabs Using Automated Sample Preparation.

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Chlamydia trachomatis is a major cause of sexually transmitted disease in humans. Sensitive and reproducible detection of *C. trachomatis* is essential to monitor and help prevent further spread of this pathogen. Our department is a NS-EN 45001 certified clinical laboratory for routine diagnostic testing of a wide range of human pathogens. We validate our in-house methods in compliance with the NS-EN 45001 standard. Molecular techniques, and especially PCR, have become an important tool for detecting bacteria and viruses in our laboratory. Real-time PCR is currently our method of choice as it provides an excellent means of multiplexing and detection. We had a need for a high throughput, cost effective diagnosis of *Chlamydia trachomatis* and in response we developed a method for automated DNA isolation and subsequent PCR detection.
The PCR combines one set of primers, a synthetic internal control and two dual labelled probes in a mastermix from Eurogentec. The internal control is longer than the *C. trachomatis* amplicon and in a very low concentration so as not to inhibit positive samples. DNA was purified from urine and swabs using a fully automated protocol on the BioRobot® M48 workstation together with the MagAttract® DNA Mini M48 Kit.
The primers were designed against *truncated inclusion membrane protein A.* 33 different bacteria, human DNA and viruses were tested against our primers. All tested negative. Using commercially quantified DNA, we were able to reproducibly detect 1 DNA copy /µl. No inhibition has been seen after testing 168 swabs and 557 urine samples.
Together with the PCR method this was compared to ROCHE's COBAS Amplicor using a panel of 282 clinical samples, were 34 (12 %) was true positives. The samples with diverging results were sent to be tested by Becton Dickinson strand displacement amplification (SDA) at an external laboratory. The in-house PCR showed 97 % sensitivity, 100 % specificity, 100% positive predictive value (PPV) and 99 % negative predictive value (NPV). The Cobas Amplicor showed 97 % sensitivity, 99 % specificity, 97 % PPV and 99 % NPV.

Based on our results we are confident that our method performs equally to the COBAS Amplicor by ROCHE.

P33
Implications of Molecular Evolution on Viral Quantification using real-time PCR based assays

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Feline Immunodeficiency Virus (FIV) is a naturally occurring lentivirus of veterinary importance that causes an AIDS- like disease in infected cats and therefore has been used as an animal model for human AIDS. A major obstacle in developing vaccines against lentiviral infections is their high mutation rate. Furthermore, mutations in primer and probe binding regions provide a pitfall for molecular diagnostics. It is therefore important to determine the genetic diversity of lentiviruses in any region where vaccination or implementation of new diagnostic techniques are planned.
In the view of the inherent retroviral sequence variability we were interested in the influence of mismatches in the primer and probe-binding region on the reaction efficiency and thus the quantification. We have demonstrated that major sequence variations of three to four nucleotides prevent detection (Klein et al. (1999) Electrophoresis 20, 291-299), while minor mismatches are tolerated, but resulted in a profound underestimation of viral loads (Klein et al. (2001) Journal of AIDS 26, 8-20).
In order to develop a reliable region specific real-time PCR assay for virus quantification in natural infected cats, we sequenced larger fragments of the *gag* and *env* genes and performed phylogenetic analysis (Steinrigl & Klein (2003) Journal of General Virology 84, 1301-1307). The results point to the existence of extensive genomic variation of FIV in Central Europe. Furthermore, a suggested North to South gradient of subtype distribution in Europe is supported by these data. Despite this mixture of different clades no clear evidence for inter-subtype recombination was found in these new strains. The mixture of FIV clades in Central Europe might mirror the increased travelling of humans and pet animals over geographical barriers like the Alps. Beside the interesting possibility to study virus evolution, these results underscore the importance of a profound knowledge of the natural occurring isolates for future vaccination and therapeutic trials using naturally infected cats. Additionally, these results provide the basis to develop region-tailored real-time PCR assays enabling reliable virus quantification in naturally infected cats. Finally, these assays enabled the monitoring of viral and proviral load during a therapeutic trial using naturally infected cats.

P35
Real-time and End-point PCR detection of *T. pallidum* DNA and RNA for clinical and research application.

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T. pallidum - is a causative agent of syphilis. The diagnostic assays of syphilis are represented with the wide range of serologic tests. The only direct test for detection of T.pallidum – Dark-Field Microscopy (DFM) - has an analytical sensitivity about 10^5 microorganisms per ml and is highly dependent of microorganisms viability. Cultivation of treponemal pathogenic strains is possible only in animals and is not available for routine use. PCR is highly sensitive and specific tool for direct diagnostic of different infectious agents. Amplification with electrophoresis detection of products create a risk of contamination and is applicable mostly for qualitative analysis. Real-time PCR has many advantages - may be used both for qualitative and quantitative analysis with greatly reduced risk of contamination.
We have developed TaqMan technology for quantitative and qualitative detection Treponema pallidum nucleic acids in different clinical material – plasma, specimens from genital ulcers and skin lesions. A pair of primers and TaqMan probes (FAM-BHQ1) were chosen for highly specific region of 16S-rRNA gene. Real-time PCR was carried out on «Rotor Gene 3000» («Corbett Research R&D», Australia). Two TaqMan probes were designed for the amplified region with different stability of internal secondary structure. Average C_t values were comparable for both probes. But there were advantages for the probe with stable internal secondary structure: higher reproducibility of C_t values, higher detection limit of amplification, higher ΔRQ value. The later was important when end-point detection was used especially of samples with low concentration of templates. So as an alternative to high expensive Real-time PCR devices a low costs dual-channel fluorescent detectors (with filters - 470/510 nm, 530/570nm) may be used for End-point analysis. Analytical sensitivity was tested on serial 10-fold dilution of DNA/RNA T.pallidum Nicols st. isolated from orchitis material of infected rabbit. Four copies of DNA targets in PCR mix gave reproducible increasing of fluorescent signal during amplification. The including of reverse transcription step prior amplification increased the sensitivity of the assay because of presence a dozen ribosomal RNA targets in microbial cells. The specificity was examined on DNA isolated from different cultivated nonpathogenic treponemal strains, Borellia spp, Leptospira spp, as well other STIs.
Clinical specimens from skin and genital ulcer lesions from patients with primary (n=16) and secondary (n=45) syphilis were examined by PCR and DFM. All samples positive in DFM were positive by PCR. For the first group PCR was positive in 100% (vs. 94% DFM), and for the second group PCR was positive in 87% (vs. 49% DFM). Using recombinant RNA and DNA calibrators with known concentrations we evaluate DNA and RNA concentration of the part of samples in Real-time PCR. All samples but one of negative DFM contained DNA in range 1.5 - 4.5 lg copies/ml. On the contrary positive DFM samples but one demonstrated DNA concentration varied from 5 to 6 lg copies/ml. Two samples with the controversial results may reflect problems with standardizing of sampling material from cutaneous lesions. Quantification T.pallidum in plasma showed that concentration of DNA varied from 1.1 to 3.1 lg copies/ml, whereas RNA concentration - 1.8 to 3.7 lg copies/ml in patient with secondary syphilis. Further optimization and standardization protocol for DNA and RNA extraction must be continued. The developed TaqMan assay for qualitative and quantitative detection of T.pallidum nucleic acids may be helpful not only for diagnostic purposes but for study microbial dissemination and persistence in animal models of syphilis.

P36

Development and validation of a real time PCR to quantify Parvovirus B19-DNA in plasma pool. A normative approach.

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Real time PCR allows the monitoring of generation of PCR product during the amplification process by measuring the increase of fluorescence in the reaction vial. Two major approaches of real time PCR quantification are currently used:
- Relative quantification, in which the target expression is determined by comparing the signal generated by the target to the signal generated by a reference gene already present in the sample omitting the need of an external standard.
- Absolute quantification, in which the unknown target concentration is calculated as an absolute value on a standard curve.
The revised Ph. Eur. Monograph Human anti-D immunoglobulin (557), which will come into force on 1st January 2004, and the revised Monograph of Human plasma S/D (pooled and treated for virus inactivation) which will be published on the supplement 4.8 of the Pharmacopoeia and which will come into force on 1st July 2004 establish 10^4 IU/ml of PVB19 DNA as the upper titre limit for the human plasma pools for the manufacturing of those products. The method, a real time PCR, was developed in order to quantify the PVB19 DNA in plasma pool samples according to an absolute quantification approach: the standard curve was calculated by plotting the threshold cycles versus the input copies number. The standard used was the B19 virus DNA BRP batch 1, with a concentration expressed in IU/ml, provided by EDQM (European Directorate for the Quality of Medicines).
The assay was carried out using Qiamp MinElute Virus Spin kit for the extraction step and in-house TaqMan PCR protocol using the ABIPrism 7900 HT SDS. The presence of an internal control prevented the risk of false negative due to the presence of inhibitors. According to ICH Guidelines Q2A and Q2B and OMCL guidelines PA/PH/OMCL (03) 38, DEF, precision, accuracy, linearity, specificity, robustness and limit of quantitation were investigated. The validated protocol was characterized by a linearity range comprised between 10^5 and 10^3 IU/ml ($r^2 \geq 0,98$)
and satisfying values of precision (CV% ≤ 17%) and accuracy (80-120%).
According to the results standard operating procedures were developed and the method was transferred to the Quality Control.

P37

Differentiation for viable and heat-inactivated *Salmonella Enteritidis* by LightCycler real-time PCR

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Rapid, sensitive and specific methods for the detection of pathogens especially in foods are necessary. These aims can be achieved by PCR and in particular by real-time PCR. However, there are some problems in respect of a minimal amount of DNA, which must be available for the detection and the impossibility of differentiation between viable and dead cells. These problems can be overcome by comparing PCR´s before and after a short preenrichment step that allows viable cells to multiply. For this reason this study is made establishing the minimal enrichment time which is achieved by investigating the growth kinetics of *Salmonella Enteritidis* DSM 9898 and its behaviour in presence of growth stimulating agents. Furthermore the significance of the difference for the crossing points of viable and heat-inactivated cells is tested.
The growth kinetics of *S. Enteritidis* was investigated with and without supplementation of ferrioxamine E and/or an “enterobacterial autoinducer of growth” up to 6 hours. Quantitative PCR on *S*. invA gene was carried out after a 4 hour incubation of viable or dead cells (initial concentrations $10^0 – 10^5$ cfu/ml). The assay was made five times. Third, various mixing ratios (100 % dead; 75 % dead/25 % viable; 50 % dead/50 % viable; 25 % dead/75 % viable; 100 % viable) from viable and heat-inactivated *S. Enteritidis* were tested (100 cfu/ml buffered peptone water) three times. The differences for the crossing points before and after the enrichment step were compared statistically by student´s t-test.
The growth kinetics of *S. Enteritidis* show that the lag-phase is overcome after an incubation time of two and a half hours. A whole incubation time of four hours enables the bacteria to reach the exponential-phase and allows to complete the test taking as little time as possible. No growth stimulating effect could be detected after supplementation with ferrioxamine E and/or an “enterobacterial autoinducer of growth” compared with unsupplemented samples. Hence, the further assays were done without supplementation. The crossing points of the not heated and the heated samples before the preenrichment were between 29 (10^5 cfu/ml) and 46 (10^0 cfu/ml). After the enrichment step the crossing points of the not heated samples were between 18,5 (10^5 cfu/ml) and 37 (10^0 cfu/ml). This corresponds to a rise of about 10 cycles through the enrichment. The crossing points of the inactivated samples stay between 29 (10^5 cfu/ml) and 41 (10^0 cfu/ml) after the incubation. The crossing points of the mixing ratios lie at about 42 before the enrichment, independently of the number of inactivated cells. After the enrichment the crossing point of the completely inactivated cells stay at 42, as expected. With the increasing number of living cells the crossing point decreases from 36 (75 % dead/25 % viable) to 31 (100 % viable). Consequently, there is a noticeable difference between viable and dead *S. Enteritidis* at a level of 100 cfu /ml. Both the comparison of viable and heat-inactivated *S. Enteritidis* and the comparison of the mixing ratios of viable and inactivated cells show a significant difference ($\alpha = 0.05$) between the difference of the crossing points before and after the enrichment step at 37 °C.

P38

Real-time and end-point PCR assay for detection of *Neisseria gonorrhoeae* in clinical specimens using dual-labeled fluorescent probes.

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The detection of Neisseria gonorrhoeae by the polymerase chain reaction (PCR) is now recognized as a sensitive and specific method of diagnosing of gonorrhea infection. However the drawback of this approach is a high risk of contamination by amplified products. Real-time PCR or end-point detection of PCR products by measuring fluorescent signal in closed tube format allow to avoid this serious problem.
We report the development of a qualitative assay designed for the detection of Neisseria gonorrhoeae in clinical specimens based on detection of specific PCR product by dual-labeled fluorescent probes. We have designed shared-stem molecular beacon probes with one arm participating in both stem formation when the beacon is closed and target hybridization when it is open. Probe specific for target DNA was labeled by Fam and probe specific for internal control DNA was labeled by Joe. BHQ1 was used as a quencher. This set of PCR reagents was used for detection of specific PCR product after amplification (end-point analysis) and for real-time PCR. End-point detection of PCR product was performed by dual-channel fluorimeter (470/510nm and 530/570nm) and real-time PCR was performed by "Rotor-Gene 3000" instrument ("CorbettResearch").
To increase the specificity of our PCR tests for detection of N. gonorrhoeae we used amplification of two targets: N. gonorrhoeae cryptic plasmid DNA and cytosine methylase gene of N. gonorrhoeae. The test was carried out in two tubes with corresponding primers and probes for these two targets. Detection limit of this assay was 500 copies/ml of target DNA both for end-point and real-time tests. This value was equal to detection limit of our conventional PCR test.
Two hundred thirteen cervical and urethral swab specimens were examined for N. gonorrhoeae with this fluorescent probe based assay in comparison with electroforesis based PCR test and with results obtained previously using culture method. Fifty six specimens were positive by end-point and by real-time tests. These results were completely concordant comparing with our conventional PCR test. Most of results were concordant with results of culture assay, except 6 specimens (5 were negative and 1 was positive by culture method). The relative sensitivity of the fluorescent probe based assay compared to culture method was 98.0% and specificity was 96.8%.
The developed fluorescent probes based assay had high sensitivity and specificity. Low risk of contamination, rapid turn-around time and cost-efficiency make the fluorescent probes based assay particularly suitable for the detection of N. gonorrhoeae in a routine clinical laboratory.

P39

Quantification of HCMV DNA in whole blood by Real-Time RotorGene-Based PCR. Comparison of two approaches of normalization.

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Quantification of the CMV DNA load in blood is necessary to predict HCMV disease development and to monitor the effectiveness of antiviral therapy. It has been demonstrated repeatedly that detection of HCMV DNA in whole blood is more sensitive than in plasma. Real-Time approach to CMV DNA quantification has several advantages such as low risk of contamination and quick results. The main problem of quantitative PCR is normalization of amplification effectiveness variability from sample to sample. There are two approaches to normalize quantification of CMV in blood. One of them based on competitive reaction with recombinant internal control (IC) DNA containing the same sites for primers annealing as amplified fragment of CMV DNA. The effectiveness of DNA extraction and amplification of competitor and CMV DNA is assumed to be equal and CMV DNA copy numbers are calculated per ml of blood. Another approach based on multiplex PCR with two sets of primers and probes for simultaneous amplification and detection of CMV DNA and human DNA. In this case the CMV DNA copy numbers are calculated per definite amount of human genomes. The aim of this study was to develop assays for quantification CMV DNA in blood by two different approaches and to compare their analytical sensitivity, intra and interassay variability and linear range. Methods. DNA was extracted from 0.1 ml of whole blood by Boom guanidine-silica method. IC DNA was added to all samples before DNA extraction. DNA was eluted with 0.05 ml of TE-buffer. Competitive Quantitative Real-Time PCR Assay (CQ PCR) was performed with primers amplified IE gene of CMV and two probes with different fluorescent dyes: Fam for CMV DNA detection and Joe for IC DNA detection. Multiplex Quantitative Real-Time PCR (MQ PCR) was performed with two sets of primers and probes. One set was designed for amplification and detection of CMV DNA and the other for amplification and detection of β-globin gene DNA. For determination of CMV, IC or β-globin DNA copy numbers in PCR tube we developed three sets of recombinant DNA calibrators. The DNA copy numbers of calibrators were determined by limiting dilution method. The HCMV DNA quantity in whole blood was expressed as genome copy number per 1 ml of blood or per 10*7 of human genomes. Standarts and Controls. To standardize the developed assays we used cell-free supernatant containing HCMV (AD169) quantified by COBAS Amplicor CMV Monitor Assay. EDTA whole blood samples from donors and immunocompromized patients were used. Results. The analytical sensitivity of CQ PCR Assay was 200 CMV DNA copies per ml of whole blood. The intra and interassay coefficient of variation for different concentration of CMV DNA in donors blood (10*3, 10*4, 10*5 copies per ml) was 12, 8, 15% and 25, 26, 38% respectively. The linear range was 500 - 10*5 copies CMV DNA per ml of blood. The analytical sensitivity of MQ PCR Assay was 350 CMV DNA copies per 10*7 human DNA copies. The intra and interassay coefficient of variation for different concentration of CMV DNA in donors blood (10*3, 10*4, 10*5 copies per 10*7 human genomes) was 8, 7, 11% and 23, 15, 9% respectively. The linear range was from 500 to 10*8 copies CMV DNA per 10*7 human genomes. Conclusions. The sensitivity and reproducibility of two developed quantitative Real Time PCR assays were equal. The linear range of MQ PCR was wider than linear range of CQ PCR Assay.

P40

Quantification of MADS box genes transcription in the orchid *Dendrobium thyrsiflorum*

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MADS box genes encode transcription factor proteins with key roles in many aspects of plant development. MADS

box genes are named after the highly conserved DNA-binding motif, initially described in the *Saccharomyces* gene *MCM1*, the *Arabidopsis* gene *AG*, the snapdragon gene *DEF*, and in the human protein Serum response factor. Phylogenetic analysis of MADS box genes isolated from multiple plant species show that it is a large family of genes consisting of several different subgroups, related to different developmental functions in plants. Transcriptional quantification of MADS box genes in plants is important in elucidating possible roles of different organs and tissues in plants. Hybridization techniques are unsuitable for this purpose due to possible cross hybridization between similar genes An alternative method, which offers high sensitivity and precision such as real-time RT-qPCR is ideal. Here we analyse the expression of MADS box genes in different tissues in the orchid *Dendrobium thyrsiflorum* using RT-qPCR.

P41

Switching between metabolic states in *Saccharomyces cerevisiae* – a gene expression profiling study

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The cellular response of yeast to glucose is both of short and long term and will ultimately adapt to the optimal fermentative growth. During the adaptation phase both the induction and repression of genes takes place. In this work three genetically modified strains of *Saccharomyces cerevisiae* with altered glucose uptake were constructed. This resulted in strains with different glucose consumption rates. Here we study the short term regulation of key genes in the metabolic pathway upon glucose stimuli. Cells were harvested during sixty minutes after glucose stimulation and gene expression was measured with SYBR Green I based real-time PCR. A mathematical model that explains the gene expression profiles has been developed and applied to the data. The short term response of most of the genes starts only a few minutes after stimulation. For some genes, the expression levels changed more than 100 times upon stimulation.

P42

Evaluation of a confirmatory PCR assay for *Neisseria gonorrhoeae*

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The Roche Cobas Amplicor PCR system is used for routine detection of *Neisseria gonorrhoeae* in clinical laboratories throughout the world. However, the Cobas *N. gonorrhoeae* assay is known to cross react with some strains of commensal *Neisseria* species. Consequently, there is a need to use a second PCR assay to confirm Cobas positive results. To date, the most common target for in-house confirmatory tests has been the cppB gene of *N. gonorrhoeae.* For the past three years, our laboratory has been using a LightCycler based cppB assay. Recently, concerns have been raised over the sensitivity and specificity of *N. gonorrhoeae* assays targeting the cppB gene. It has been found that some *N. gonorrhoeae* strains lack the cppB gene whereas some commensal *Neisseria* strains contain the cppB gene. In this study, we used our LightCycler cppB assay to test a panel of Australian *Neisseria* strains to determine if such problems existed in our local population. In total, 136 local bacterial isolates, including 80 *N. gonorrhoeae* and 56 non-gonoccocal isolates, were used in the study. Of the 80 *N. gonorrhoeae* isolates tested, seven were negative by the cppB-LC assay. This shows that *N. gonorrhoeae* strains lacking the cppB gene are present in our population. In addition, the cppB-LC assay cross-reacted with three *N. meningitidis* isolates showing that the cppB gene is also not specific to *N. gonorrhoeae* in our population. Overall, the results show that the CppB LightCycler is susceptible to both false-negative and false-positive results when used on our population necessitating the need to find a new *N. gonorrhoeae* confirmatory test for the Roche Cobas Amplicor system.

P45

Quantification of the expression of the chitinolytic enzyme encoding genes *ech30, ech42* and *nag1* in *Trichoderma atroviride* P1 under varying growth conditions using a real-time RT-PCR assay

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The quantitative expression and regulation of the chitinase-encoding genes *ech30*, *ech42* and *nag1* in *Trichoderma atroviride* P1 with 0.1 % and 3 % glucose as carbon source and ammonium acetate as nitrogen source at no (0 mM), low (10 mM) and high (100 mM) levels were studied with and without colloidal chitin using a real-time RT-PCR assay. Twelve media combinations, 3 time intervals and 2 replications were used to study the expression and regulation of *ech30*, *ech42* and *nag1* in *Trichoderma atroviride* P1. The expression of *ech30*, *ech42* and *nag1* was regulated by the interaction of nitrogen, glucose and chitin under different growth conditions. The highest and earliest expressions of *ech30*, *ech42* and *nag1* were induced by glucose and nitrogen starvation. In contrast, high glucose and ammonium acetate concentrations repressed the expression of all 3 genes. The repressive role of glucose and nitrogen was confirmed by multivariate and statistical analysis. A negative relationship between chitin and *ech30* expression was found in all the cultures studied, in contrast to a generally positive relationship found between chitin and both *ech42* and *nag1*. Alternative nitrogen sources and autolysis in *T. atroviride* P1 are discussed.

P65

A real-time RT-PCR SYBR Green-I assay for detection of porcine reproductive and respiratory syndrome virus

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Porcine reproductive and respiratory syndrome virus (PRRSV) is the cause of Porcine reproductive and respiratory syndrome (PRRS), a severe disease in pigs with great economical consequences to swine producers worldwide. PRRSV belongs to the *Arteriviridae* family. The genome of PRRSV is a single-stranded positive sense RNA molecule of approximately 15 kb in length, containing at least eight open reading frames (ORFs). PRRSV isolates are divided into European (EU) and American (US) types of PRRSV based on substantial antigenic and genetic variation.
We have developed a real-time RT-PCR strategy based on SYBR Green-I chemistry with the aim to detect PRRSV in animal samples. The strategy is based on a previously published conventional RT-PCR method (Oleksiewicz et al. 1998, Vet.Microbiol. 64:7) and relies on amplification of whole viral genes: ORF7, which will detect all known types of PRRSV, and ORF5, for typing of isolates. We have applied this real-time RT-PCR assay to study the susceptibility of ducks to PRRSV. PRRSV is well known to infect pigs. In addition, results from a previous study has suggested the involvement of avian species in the epidemiology of PRRS (Zimmerman et al. 1997, Vet.Microbiol. 55:329). In order to investigate the potential of mallards as reservoirs for PRRSV, we inoculated 2 groups of mallards by the nasal/oral route with isolates of EU and US types of PRRSV, respectively. Tissue samples and cloacal swabs were taken from inoculated animals at various times up to 35 days post inoculation. We have tested these samples for the presence of PRRSV by the real-time RT-PCR assay. Results from these experiments and technical issues related to establishment of the real-time PCR assay will be presented.

P132

Real-time PCR Detection of Orthopoxvirus DNA and Identification of *Variola Virus* DNA by Melting Analysis.

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Although variola virus was eradicated by the WHO vaccination program in the 1970's, the diagnosis of smallpox infection has attracted great interest in the context of a possible deliberate release of variola virus in bioterrorist attacks. Obviously, fast and reliable diagnostic tools are required to detect variola virus and to distinguish it from orthopoxviruses that have identical morphological characteristics, including vaccinia virus. The advent of real-time PCR for the clinical diagnosis of viral infections has facilitated the detection of minute amounts of viral nucleic acids in a fast, safe and precise manner, including the option to quantify and to genotype the target reliably. Here we present a set of 4 hybridization probe-based real-time PCR assays for the specific detection of orthopoxvirus DNA. Melting analysis following PCR enables the identification of variola virus by the PCR product's characteristic melting temperature permitting the discrimination of variola virus from other orthopoxviruses. In addition, an assay for the specific amplification of variola virus DNA is presented. All assays can be performed simultaneously in the same cycler and results of a PCR run are obtained in less than one hour. The application of more than one assay for the same organism significantly contributes to the diagnostic reliability, reducing the risk of false negative results due to unknown sequence variations.

P133

Absolute quantification of pathogens in food samples using floatation as a novel sample treatment method prior to qPCR.

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Since the development of real-time PCR, it has been widely applied in different fields. Also in the area of food microbiology and the detection of food-borne pathogens it has gained popularity. However, dealing with qPCR and absolute quantification in food samples, challenges have to be faced: i.e. the increased sensitivity to PCR inhibitors, the necessity not to influence the original amount of target cells in an uncontrolled way, and risks concerning false-positive qPCR results due to detection of DNA from dead cells. This has lead to a great demand for sample treatment strategies that can overcome these obstacles. We have developed and demonstrated the use of a novel sample treatment called floatation, which in combination with qPCR is used for the absolute quantification of *Yersinia enterocolitica* and *Campylobacter* spp. in several food and clinical samples. Floatation is a new variant on traditional buoyant density centrifugation. Results showed that after floatation the sample matrix and the background flora (BGF) could be separated from the target pathogen in such a way that PCR inhibition was minimised to levels comparable to that of purified DNA in Millipore water. Applying floatation to meat juice samples, containing natural BGF and spiked with different concentrations of *Y.enterocolitica*, showed that absolute quantification of *Y.enterocolitica* was possible down to levels of 4.2×10^3 CFU / ml even if an additional 10^6 CFU / ml BGF were added. Furthermore, the design of the floatation setup ensured that false-positive results from artificially added free target DNA (simulating DNA released from dead cells) up to levels of 2.1 µg / ml did not occur. Also following dying cell cultures by viable count, and floatation followed by qPCR, revealed that after floatation, in contrast to direct qPCR, the risk of false-positive results was greatly reduced.

Poster Session: Normalization & Standardization & Quality control

P43
Design and testing of glyceraldehyde-3-phosphate dehydrogenase (GAPD) primers to avoid pseudogenes amplification in Real-Time PCR.

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In relative quantification studies of gene expression, housekeeping genes are often used to normalize data. This is needed to evaluate the relative expression of a target gene transcript in comparison to a reference gene transcript.
Glyceraldehyde-3-phosphate dehydrogenase (GAPD) is a glycolyitic enzime active in all mammalian tissues and it is frequently used as an endogenous control in expression studies by using Reverse Transcription Polimerase Chain Reaction (RT-PCR).
The functional locus maps to human chromosome 12p13, but several GAPD-related sequences, as know processed pseudogenes, GenBank homologous sequences and computationally predicted sequences are present along the human genome. Due to the high level of GAPD-related sequences it is very important to avoid genomic DNA amplification when GAPD is used in mRNA quantification.
GAPD mRNA sequence was analyzed with nucleotide-nucleotide BLAST against the non-redundant database avaible at the NCBI website. A wide number of homologous sequences including 11 designated pseudogenes and about 70 similar sequences and computationally predicted sequences were found and analized with Genome Browser and Map Viewer tools.
We out lined a couple of primer able to avoid genomic DNA amplification and consequently also that of the pseudogenes, and performed a excellent standard curve for real-time PCR with Syber Green.
These new designed primers are a useful, chip and commercially not avaible alternative to probe technologies that carry out specific and reproducible data in expression studies.

P44
Development of a Quantitative Real-Time Polymerase Chain Reaction (QRT-PCR) Assay using Hybridisation Probes to Detect the Fungus *Aspergillus*

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Aim: Immuno-suppressed patients are highly susceptible to *Aspergillus* infections, which could be potentially fatal, thus early and accurate detection of *Aspergillus* infection is essential for the proper diagnosis and treatment of *Aspergillus*-related diseases. We have developed a Quantitative Real-Time Polymerase Chain Reaction (QRT-PCR)-based assay that not only accurately detects but also quantifies the amount of 18S ribosomal DNA (rDNA) of *Aspergillus* present, within an hour.
Methods: Fluorescence-labelled hybridisation probes and primers with specific nucleotide sequences homologous to a conserved region in the 18S rDNA of *Aspergillus* were designed. The assay was optimised and calibrated to quantify the amount of starting DNA. Presence of *Aspergillus* species yields a 172bp amplicon on the LightCycler® (Roche) instrument. Both analytical and diagnostic sensitivity and specificity were determined, and various fungal cultures, spiked clinical samples (blinded to the analyst) and paraffin-embedded tissues (PET) showing the characteristic morphologic appearance of fungal infections were used to validate the assay.
Results: The assay was 100% accurate in detecting various *Aspergillus* species in cultures, and in all 5 samples containing 100 fg of genomic DNA or 2-10 cells. Using this assay, one can detect down to 10 fg of DNA, with 60-80% consistency. There was no cross-reactivity with clinical *Candida* isolates or with *Cryptococcus neoformans* in fresh tissues. However, we achieved relatively less success when we applied the assay to the detection of *Aspergillus* in PET, which may be partly due to possible misclassification of aspergillosis based on histomorphological criteria (i.e., false positives caused by *Crytococcus neoformans, Fusarium* spp and other fungi). Based on 31 spiked clinical blood samples, we showed that when the following set of criteria are fulfilled to indicate the presence of *Aspergillus*: a melting peak between 63.2°C and 64.9°C, the test gave a 100% diagnostic sensitivity and a 100% diagnostic specificity.
Conclusion: This QRT-PCR assay is rapid, accurate, and highly specific and can be adapted for diagnosis and monitoring of *Aspergillus* infections in immuno-compromised hosts.

P46
Stability of six housekeeping genes in nine ovine tissues and its application to the relative quantification of prion protein gene expression

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Real-time RT-PCR is a suitable tool for studies on differential gene expression. This technique provides the high accuracy and reproducible results necessary for mRNA quantification experiments. In addition, real-time RT-PCR allows the normalisation of a target gene expression with stable housekeeping (HK) genes, making it an ideal tool for this kind of analysis where samples of a variety of sources and different nature are analysed. Most studies on gene expression have focused on human or experimental animal models, however, there are no studies of this kind in sheep. This work reports the analysis of the stability of six HK genes among nine ovine tissues and, the relative quantification of prion protein (PrP) gene mRNA using real-time RT-PCR SYBR Green detection. Selection of the most suitable HK genes was assessed on the basis of the gene expression stability measure (M) and the pairwise variation between the normalisation factors ($V_{n/n+1}$) obtained from the Visual Basic Application geNorm v 3.2. Taking into account the heterogeneousness of the tissues

analysed and the obtained data, we selected the four most stable HK genes ACTB, RPL19, GAPD and G6PDH and discarded the less stable genes YWHAZ and SDHA. The normalisation factor for each tissue was calculated by the geometric mean of the raw values obtained from the four selected genes. In the analysed tissues, the relative expression of PrP gene showed a wide range of values with approximately 24-fold expression difference between cerebrum and ganglia. The sequence of PrP gene expression from highest to lowest was cerebrum, cerebellum, obex, spinal cord, terminal ileum, tonsil, spleen, liver and ganglia. In summary, these results provide the first data on the stability of six ovine HK genes useful for further expression analyses, and present preliminary data on PrP gene expression.

P47

Analysis of the expression stability of *Actinobacillus pleuropneumoniae* housekeeping genes during *in vitro* growth under iron-depleted conditions by Real-Time quantitative PCR

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The aim of the present investigation was to develop and test a sensitive and reproducible method for the study of gene expression in the porcine lung pathogen *Actinobacillus pleuropneumoniae* by Real-Time quantitative PCR and to evaluate a number of suitable internal controls. The expression stability of five genes involved in basic housekeeping, *glyA*, *tpiA*, *pykA*, *recF*, and *rhoAP* from bacteria grown in medium with and without sufficient iron, respectively, was measured during *in vitro* exponential and stationary growth. The stability of expression among the housekeeping genes was evaluated by calculating the mean pair-wise variation for a gene with all other tested control genes. Next, five genes of interest were compared and the most stably expressed housekeeping genes, *glyA*, *pykA* and *rhoAP* were used as internal controls for normalization. The compared genes were *tpbA* (transferrin binding protein) and *tolQ* (inner membrane protein), which are transcribed on a single polycistronic mRNA and which both have been shown to be expressed under iron-limiting growth conditions only. Also included in the analysis were *fhuD* (ferric hydroxamate uptake protein), *hsp-60* (heat shock protein) and *luxS* (a gene responsible for the production of autoinducer 2, which, in some bacteria, seem to function as a quorum-sensing signal under iron limitation). After correcting the raw values, the level of *tbpA* and *tolQ* expression in *A. pleuropneumoniae* showed rapid and significant up-regulation in the exponential growth phase under iron restricted conditions as compared to bacteria grown in medium with sufficient iron. For the remaining three genes no significant regulation in any of the growth phases was seen. The observed expression patterns of *tbpA* and *tolQ* were consistent with previous observations and this experiment therefore lends further support to the use of the genes *glyA, pykA* and *rhoAP* as internal controls for future gene expression studies in *A. pleuropneumoniae*.

P48

New tools for quality control in real-time PCR experiments

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Real-time PCR experiments allow the fast and efficient assessment of the expression of single genes. The reliability of the real-time PCR data is largely dependent on the quality of the assay itself as well as on the quality of the template and the specificity of the PCR products. Lab-on-a-Chip technology allows the quality assessment of RNA and PCR samples at different steps within the real-time PCR workflow. Applied on an analytical platform, the degradation state of RNA samples can be monitored allowing RNA integrity assessment at the beginning of an experiment. To improve the standardization of the RNA QC process, a software algorithm has been developed for the classification of RNA samples. First test indicated that analyses by the software are more reliable than the manual evaluation of RNA integrity by considering the ribosomal ratio of the samples. Furthermore, in addition to melting curve analysis, the high resolution of the Lab-on-a-chip-technology provides the opportunity to verify the specificity of PCR products at the end of an experiment.
In summary, here we provide data how quality control can be implemented in a real-time PCR study to improve the reproducibility and reliability of gene expression experiments.

P49

Search by cluster analysis for steadily expressed genes with application as normalisation index in real-time RT-PCR.

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Cluster analysis is a tool often employed in the micro-array techniques but less in the real-time PCR. The correlation analysis and the clustering procedure VARCLUS of the SAS software applied on gene expression data obtained by real-time RT-PCR are shown to be a powerful tool in search for steadily expressed genes. These genes can, then, serve as a robust indexed standard for a gene-expression normalisation. The constructed SAS macro, based on this procedure, divides a set of genes into hierarchical clusters. PROC VARCLUS tries to maximize the sum across clusters of the variance of the original expression data that is explained by the cluster components. Instead of the Euclidian distances a correlation coefficient is taken as a dissimilarity measure. The dissimilarity measure is made robust using a rank order correlation coefficient rather then parametric one. There is no need for an overall probability adjustment as in scoring methods based on repeated pair-wise approaches.
Some simple approach in finding similarly stable genes, ignoring the imaginary boundary between unregulated housekeeping genes and regulated genes, is wanted, that would group genes into clusters based on a robust distribution-insensitive similarity measure.
Clustering approaches have been frequently adopted on micro-array data to disclose families of co-regulated genes.

A similar pattern of expression indicates co-regulated genes. Some genes, however, can remain untouched by the experiment. If more such unregulated genes are compared, they, as well, show a similar pattern. This similarity is given by the stabile expression ratio between any two of the genes. Therefore a correlation coefficient between two unregulated genes indicates similarity. Where the sampling, extraction procedure, RT reaction, storage and the PCR performance was affected by error, all genes achieve some common erroneous shift. This shift produces some common visible pattern only in genes that are not biologically regulated, because any biological regulation would mask the minor erroneous pattern.
The success of the method of cluster analysis depends on how well its underlying model describes the patterns of expression. Based on above idea, the herein suggested cluster analysis associates genes based on similar correlation patterns as given by the correlation matrix. Genes with different expression levels but correlating well due to steady expression ratio are clustered together. The Euclidean distances cannot be taken as a measure of dissimilarities here, because the levels of expression can be very different.
The real-time PCR yields the so called crossing points or threshold cycles; those are the fundamental quantitative units. This data shows skewed distribution and heterogeneous variance. The Gaussian distribution is only rarely given, therefore, the here proposed method clusters genes based on the non-parametric Spearman correlation coefficient, making the method distribution-insensitive.
If a distinct cluster contains predominantly known housekeeping genes, its genes can be applied for standardisation purposes in form of geometric mean.

P50
Comparative Quality Assessment (CoQA) for real-time PCR

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The exponential nature of PCR makes DNA quantification by real-time PCR sensitive to differences between the efficiencies of the compared samples. Therefore, the most common quantification methods require similar efficiencies for proper comparison. Based on this prerequisite, we suggest a Comparative model for Quality Assessment (CoQA) of real-time PCR. In CoQA, a set of experimental samples is considered of low quality if the ratio: variance of efficiencies of experimental samples / variance of efficiencies of high quality standard samples is > 3-4. Such standard samples may consist of purified PCR product, which shows the highest reproducibility in quantification. Using an F-test we tested the ratio between the variances of efficiencies of standard and experimental samples from 30 sets of 5 replicate each (same experiment on 5 animals). 8 sets of low quality quantification were identified. The variance of the DNA amounts quantified in the low quality sets were significantly higher ($P<0.01$) than the variance of the other sets. These findings suggest that the ratio of efficiencies variances could be used to draw attention of the real-time PCR user to suspicious samples.

P51
Real-time PCR survey

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The Nucleic Acids Research Group (NARG) of the Association of Biological Resource Facilities (ABRF) conducted an on-line survey designed to "take the pulse" of the real-time PCR international community. Respondents were asked questions concerning the type of facility, personnel, instrumentation, types of assays, templates, extraction methods, controls, analysis, etc. Additional questions for those that run core facilities that offer real-time PCR as a service were included. Participation was anonymous. Results provided insight into the current state of real-time PCR. Results will be freely available to all on-line.

P52
Amplitude normalization in real time PCR data processing

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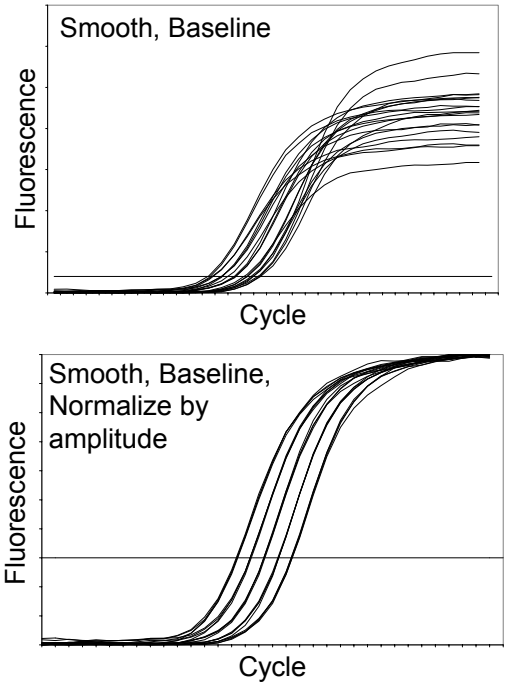
Optimal procedures for data processing are essential to derive maximal output from real time PCR (RT PCR). The use of amplitude normalization to improve precision of RT related calculations is described below.
A simple computer simulation predicts the same position of the plateau in different samples for the starting conditions normally recommended in real time PCR. Notable differences that are often observed in level of the plateau (even in replicas) may not be a specific PCR-related signals and should be filtered out as a noise. Commonly used filtering procedures (*i.e.* smoothing and baseline subtraction) do not apply to this kind of noise. To address this issue the use of amplitude normalization is suggested *i.e.* division of each reading in the cell by the maximal reading in this cell through the run.
This procedure was used while measuring expression of 6 genes in 42 biopsies of breast cancer. RNeasy Mini Kit (Qiagen), SuperScript RT (Invitrogen), QuantiTect SYBR Green PCR kit (Qiagen) and Opticon Monitor PCR machine (MJ Research) were used. PCR was performed in quadruplicates, without passive dye normalization, with standard curve on each plate. Raw plate readings exported by Opticon Monitor software was analyzed with home made application developed using VBA for Excel (Microsoft).



2 fold serial dilutions of standard with and without amplitude normalization

Amplitude normalization decreased average dispersion in replicas by 2 to 7 times. In a typical example presented above amplitude normalization decreased average coefficient of variation (CV) for crossing points on the 96 plate from 1.2% to 0.3% and increased coefficient of determination (R2) from 0.965 to 0.999. In the vast majority of cases while using amplitude normalization CV was below 0.5% and R2 was above 0.99. Propagating to the final results amplitude normalization notably decreased dispersion but usually did not change significantly the mean values. Amplitude normalization improved parameters only when the true replicas where used, mixing all components before dispensing samples to the plate. When "replicas" were made by adding cDNA separately from other pre-mixed components the dispersion was usually higher and amplitude normalization did not improve results significantly.
These data show that amplitude normalization remarkably increase precision of RT PCR providing correct procedures were used during the real PCR setup.

P53
Performance of different qPCR approaches: Comparing accuracy and reproducibility of conventional 5' nuclease probes, short LNA enhanced probes and SYBR Green

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The increasing interest in homogenous assays over the last decade has stimulated the development of a number of different qPCR technologies to determine gene expression levels in cells. Two of the most frequently used approaches are 5' nuclease probes (e.g. TaqMan™) using a sequence specific fluorescent detection probe and SYBR Green that use an non-sequence specific intercalating dye. We have compared these conventional approaches to a novel type of short LNA enhanced 5' nuclease probes. The novel probes differ from other q-PCR probes in containing duplex stabilizing LNA™, which reduce the probe length and enable detection of frequently occurring "gene-tags" of 8-9 nt's. Each probe thus target thousands of sites in the human transcriptome and even a small library of only 90 short probes (the Human Probe Library) will detect 99% of all human transcripts. Results will be presented showing the sensitivity, accuracy and reproducibility of the relatively short probes in the Human Probe Library in comparison to Assay-on-Demand™ 5' nuclease probe kits from Applied Biosystems and to SYBR Green based assays. Examples where SYBR Green based qPCR assay failed will be discussed. Compared to generally available transcript-specific dual-labelled probes, the probe library provides flexibility and cost-effectiveness. Compared to the economical SYBR Green based real-time PCR detection assay the Human Probe Library provide sequence specific hybridization dependent fluorescence, thus removing the problem of primer-dimer derived fluorescence and mispriming.

P54
The influence of pigmentation and evaporation on Quantitative PCR

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Intercalating dyes such as SYBR® Green I are commonly used in quantitiative PCR. SYBR® Green I offers a simple and economic approach to DNA quantification whereby the fluorescent signal increases in direct proportion to the accumulation of double-stranded DNA products. In order to ensure accurate quantification of low copy-number sequences and rare targets, it is necessary to ensure that template DNA does not stick to pipette tips and tubes during reaction preparation. This can be achieved by using low-DNA binding plastics. Results show that using a white PCR plate instead of a clear plate to perform the assay can further increase sensitivity. Clear PCR plates allow light to be transmitted unevenly through the tube causing increased variability of the reflected signal. However, white plates reflect light from the interior well wall, preventing transmission through the tube ensuring a uniform signal is detected. As a result, rare targets can clearly be distinguished from background noise. Additionally, results indicate that sample evaporation can cause a delay in Ct and reduced end-point values. By ensuring a tight seal and combining the use of white PCR plates with technologies shown to decrease DNA binding to plastics, the sensitivity of a SYBR® Green I assay may be further enhanced.

P55
Integration of Comparative Quality Assessment (CoQA) for real-time PCR into Kinetic Outlier Detection (KOD)

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The exponential nature of PCR makes DNA quantification by real-time PCR sensitive to differences between the efficiencies of the compared samples. Therefore, the most common quantification methods require similar efficiencies for proper comparison. To verify this requirement we have developed two methods (i) Kinetic Outlier Detection (KOD) that compares the similarity of a sample-specific efficiency to a set experimentally relevant sample and (ii) Comparative Quality Assessment (CoQA) that assess the similarity of set of samples by comparing their spread of efficiencies to the spread of efficiencies of high quality samples. Here we combine the two methods and detect outlier samples in relative quantification by comparing a test sample efficiency to the mean value of the other experimental samples using the standard deviation of efficiency of high quality samples. We test the combined method and found moderate correlation between the spread of efficiencies and the spread of quantities in sets of replicate samples (same experiment different animals). Outliers' exclusion reduced the spread of the results. In conclusion we recommend considering biological outliers that are also kinetic outliers as suspected. The treatment of these samples may involve repetition of previous steps of the quantification, possibly with additional step for removal of PCR inhibitors.

Poster Session: Transcriptomics & Expression profiling

P56
qRT-PCR as a tool for studying the expression pattern of Wnt genes, their receptors and their secreted antagonists in the Mouse Embryo

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Many signaling pathways are active during the development from the fertilized egg to the mature organism. One of these pathways is the extensively studied Wnt pathway. Wnts found throughout the animal kingdom, are secreted proteins and until today, 19 members of this family are known in the mouse. Up to now 10 of the Wnt receptors have been identified (Frizzleds) and 11 of their antagonists (Frzb-1, sFRPs, Dkk,…)
They are not only essential signals during the development of the mouse -they participate in gastrulation, neurulation and organogenesis- but they are also involved in renewal and differentiation of stem cells (Embryonic Stem cells and adult stem cells), differentiation of organs and in cancer and hair growth.
Wnt-1 was discovered the first and appeared to be involved in tumor growth. Later on by performing knock-out studies, it was revealed that Wnt-1 is also involved in the development of the midbrain and neural stem cells. Similar loss of function studies have unraveled the functions of many members of the Wnt family. Wnt-3 for example was shown to be necessary for the development of the embryo as gene activation resulted in gastrulation arrest and subsequent lethality. In this line it was show that beta-catenin, a downstream factor of the Wnt-pathway, is also necessary for initiating gastrulation. Another example is the cooperation of Wnt-1 and Wnt3a in muscle formation.
Here we present qRT-PCR data, showing the expression patterns of all 19 Wnt genes, their 11 antagonists and their 10 Frizzled receptors in the developing mouse embryo. Their expression was studied from the blastocyst stage to the 8.5 dpc stage by using Sybr Green qRT-PCR. Several patterns were confirmed by *in situ* hybridizations.
With this approach we hope to reveal specific Wnt expression patterns during development that could contribute to discovering new functions of the existing Wnts.

P57
Telomerase reverse transcriptase expression in twenty-three acute promyelocytic leukemia patients: relative quantification by real-time polymerase chain reaction.

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Telomerase activity is increased in almost all malignant hematological disorders, including acute myeloid leukemia (AML), however its role in disease pathogenesis and progression is still undefined. It is composed of an RNA subunit (TERC) and a rate-limiting catalytic protein subunit (TERT). Its function is to maintain nucleoprotein structures that constitute the ends of eukaryotic chromosomes (the telomeres). Telomeres play a crucial role in many cellular functions and their erosion with cell senescence prevents tumor formation by limiting cell life span. However not all senescent cell die, as rare cells in which telomere length is maintained due telomerase activation may survive and eventually cause cancer. In the present study 23 AML-M3 patients enrolled in the AIDA protocol and treated at our Institution were analysed for TERT expression on clinical diagnosis and during the follow-up. The aim of our study was to establish whether TERT expression correlated with any clinical parameters and with relapse. A relative quantification of TERT transcripts was performed with a real-time polymerase chain reaction using SybrGreen I as double-stranded DNA-binding fluorescent dye. In order to obtain a standard curve for real-time quantification we constructed a standard curve by using serial dilutions of total RNA from K562 cells. TERT expression levels were normalized to ABL mRNA expression and calibrated on K562. Dissociation curve analysis were employed to

demonstrate the specificity of TERT amplification. Thirteen patients were males and 10 were females, their mean age was 39.7 years (range 19-67). All the 23 patients achieved a complete remission (CR) after a median time of 35 days (range 30-60) and their median follow-up is now 28 months (range 4-92). Seventeen patients are still in an un-maintained CR after a median follow-up of 24 months (range 3-90), while 6 have relapsed after a median time of 17 months (range 9-35) from first CR. All of these last patients but one are now in a second CR. The Spearman's test found a statistically significant correlation between TERT expression levels on clinical diagnosis and a high WBC count (p=0.03), an association also seen in one relapsed patient. As in our M3 series hyper-leucocytosis was strictly associated with an internal tandem duplication (ITD) of the FLT3 gene, we examined this last mutation in relation to TERT transcript levels and we observed that mean TERT transcript levels were 6.4 (range: 0.7-9.9) in patients with a high WBC and FLT3-ITD, while they were 4.9 (range: 0.3-14.7) in patients with normal WBC and without FLT3-ITD. In contrast, when a regression model for repeated measurements was applied to TERT expression levels during the follow-up, no significant variation of TERT expression over time was detected. In order to establish whether TERT expression levels at clinical diagnosis were predictive of relapse, patients were subdivided in two groups with low and high TERT transcript levels. Patients with low TERT transcript levels at clinical diagnosis had a relapse rate of 4.7% (95% confidence intervals, CI =0.6-33), while those with high TERT transcript levels had a relapse rate of 15.9% (95%CI =6.0-42). Therefore, these last had a 4.3% relative risk (95% CI =0.48-39) of developing relapse (p= 0.14). When we evaluated relapse rate at one hundred-eighty days after diagnosis, patients with low TERT transcript levels had a relapse rate of 6.8% (95%CI= 0.9-48), while those with high TERT transcript levels had a relapse rate of 21.7% (95%CI= 7.0-67). Patients with a high TERT expression had a 3.5% relative risk (95%CI=0.37-34) of developing relapse (p=0.23). In conclusion our study shows a trend toward an increased risk of disease recurrence in patients with high TERT levels.

P58
RGS2 expression and intracellular calcium mobilization in fibroblasts from hypertensive patients

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Backgound: RGSs (regulators of G-protein signaling), a family of GTPase-activating protein, increase the deactivation rate of heterotrimeric G proteins. RGS2 is a negative regulator of G□q, which mediates the action of angiotensin II. Knock out mice for RGS2 gene exhibit a hypertensive phenotype and a prolonged response to vasoconstrictors.
Aim: To compare RGS2 expression and Ca^{2+} mobilization induced by angiotensin II in fibroblasts from normotensive subjects and hypertensive patients.
Methods: Skin fibroblasts from normotensives (n=10,aged 37±3, BP 125±2/74±2 mmHg), and hypertensives (n=11, aged 46±2, BP165±2/100±1 mmHg) were cultured and used after three passages. Quantitative mRNA RGS2 expression was performed by real-time quantitative RT-PCR using specific fluorescent TaqMan probes in a multiplex reaction for RGS2 and the housekeeping GAPDH gene. Spectrofluo-rophotometric free Ca^{2+} measurement was performed in monolayers of 24-hour serum-deprived cells, using FURA-2 AM, as fluorescent probe.
Results: mRNA RGS2 expression was significantly lower in fibroblasts from hypertensive patients in comparison to normotensives (A.U. 0.65±0.01 *vs* 0.80±0.01 RGS2/GAPDH, respectively, $p<0.05$). Resting intracellular Ca^{2+} level and angiotensin II-stimulated intracellular Ca^{2+} peak were higher in fibroblasts from hypertensives compared with those from normotensives (93±10 *vs* 54±2 nM, respectively, $p<0.05$, and 199±8 *vs* 128±8 nM, respectively, $p<0.01$).
Conclusion: In fibroblasts from hypertensive patients RGS2 expression is reduced, and Ca^{2+} mobilization induced by angiotensin II increased. Therefore, these results suggest that a low expression of RGS2 may account for increased G protein-coupled signaling in human hypertension.

P59
Use quantitative real-time RT-PCR to measure innate immunity

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We are using real time quantitative PCR to study the effect of *Mycobacterium tuberculosis* (Mtb) sonicate on Toll like receptor mRNA expression in the human leukemia monocytic cell line THP1. Our aim is to investigate the role of innate immunity in Tuberculosis. The first stage in our study was to define our normalisation strategy. Normalisation distinguishes gene expression differences from differences due to experimental error, such as varying mRNA concentration, reverse transcription efficiency etc. This can be done by input cell count, measuring total RNA or using a reference (house keeping) gene. We decided to use the reference gene method as it is simple to perform and controls for both extraction and reverse transcription error. The initial stages of our study were to 1) identify a suitable reference gene for human THP1 and 2) characterise the Toll like receptors (TLRs) expressed by THP1 in response to Mtb sonicate. We examined the effect of Mtb sonicate on 3 potential reference genes over 48 hours, standardizing relative to mRNA concentration measured using the bioanalyzer. All potential reference genes had standard deviations of less than 2 fold. However Human Acidic Ribosomal Protein (HuPO) varied the least with a standard deviation that differed from the mean by 1.33 fold. In the next stage of the study we established that Mtb sonicate upregulates genes encoding TLR 2, 4 and 6, which confirms results from other groups. The future study will focus on the role of innate/acquired immunity induction through toll like receptors and the possible role of IL-4 on modification of TLRs expression.

P60

Evaluation of HR-HPV-E6-E7 mRNA in histologically negative sentinel lymph nodes of cervical carcinoma patients as a predictive marker for recurrence.

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Background: Close to 20% of patients with cervical cancer and histopathologically negative lymph nodes (pN0) experience recurrent disease. The detection of tumor associated molecular markers in sentinel lymph nodes may proof to be a valuable prognostic tool. Methods: In this ongoing study sentinel lymph nodes of 95 high-risk HPV positive cervical cancer patients with FIGO Stage I (n=69), FIGO stage II (n=25), and FIGO stage III (n=1) were tested for HPV oncogene transcripts E6-E7 by a RT-nested-PCR approach. Furthermore sentinel nodes of HPV16 positive patients were analysed by quantitative real time PCR for E6-E7 transcripts and the epithelial specific cytokeratin CK19. Results: 21 of 95 patients had histologically positive sentinel lymph nodes. These lymph nodes were also positive for HPV mRNA. Of 74 patients with histologically negative sentinel lymph nodes 6 patients had histologically positive lymph nodes at other sites. The sentinel lymph nodes of 3 of these patients were also positive for HPV mRNA. Of 68 patients with pN0 status 13 patients (19.1%) had HPV mRNA positive sentinel lymph nodes. Discussion/Outlook: HPV mRNA is a highly sensitive and specific marker for the detection of disseminated tumor cells in lymph nodes. Clinical follow up data of our patients in relation to the level of HPV oncogene expression will be presented at the meeting.

P61

The *in vivo* effects of a Mycophenolic Acid (MPA) treatment on the cytokine expression in sheep leucocytes, using an efficiency corrected relative quantification model in real-time RT-PCR.

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Mycophenolic acid (MPA) is an active secondary metabolite from *Penicillium roqueforti*. It is frequently found in contaminated silages for animal feed. MPA is known to have immune suppressive effects and is therefore used in human medicine as an anti-proliferative agent and for immune suppression after organ transplantation. In the present study were investigated the *in vivo* effects of a long term MPA administration (9 weeks) on cytokine mRNA expression in sheep leucocytes. Various qRT-PCR assays for immune relevant cytokines were developed: tumor-necrosis-factor alpha (TNFα) and four interleukins (IL1α , IL1β, IL2 and IL6). For quantification we validated and applied a recently describe method using an efficiency corrected relative quantification model using REST©. Expression data were normalised by the non-regulated mRNA expression of the housekeeping gene β-actin. The cytokines were compared between dose- and time-effect over 9 weeks. No effects were found on leucocytes total RNA contents. Each factor exhibited an individual mRNA expression pattern. qRT-PCR relative quantification showed a significant 4-fold up-regulation of TNFα mRNA after 1 week ($p<0.05$) in the MPA treated group. IL1α showed a continuous 6-fold down-regulation ($p<0.01$) between week 2 and 5, whereas IL1β exhibit a 2-fold down-regulation at the end of the trial. The remaining cytokines IL2 and IL6 had a constant expression pattern. Both IL1 subtypes are responsible for the initial B-cell activation and proliferation and therefore a down-regulation might have severe immune suppressive effects.

P62

Analysis of differentially expressed genes in kidneys of PEPT2 knockout mice

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Transporters for di- and tripeptides that belong to the proton-coupled oligopeptide transporter family (POT) are found in prokaryotes and eukaryotes. In mammals two different systems have been identified. PEPT1 is mainly expressed in the small intestine where it mediates the absorption of dietary peptides. PEPT2 on the other hand shows widespread expression within the organism. The highest expression level of PEPT2 is found in the kidney, where it may contribute to the renal reabsorption of filtered di- and tripeptides. To study the physiological role of PEPT2 we have generated a knockout mouse line that lacks a functional PEPT2 protein. Differential gene expression in knockout versus wildtype mice was analysed by a 20K cDNA microarray with mice kidney mRNA trascribed into cDNA. Of each of five *Pept2*$^{-/-}$ mice kidney cDNA samples were hybridised four times against a *Pept2*$^{+/+}$ kidney cDNA pool. Differentially expressed genes were identified by pattern analysis and confirmed by quantitative real-time PCR. To validate that differential gene expression is due to the lack of the peptide transporter PEPT2, kidney mRNA of mice kept on a low, normal or high protein diet (housed in metabolic cages) was analysed by quantitative real-time PCR. Our results show that genes that displayed strongest differential expression in the cDNA microarray experiment are likewise differentially expressed in mice kidney supporting a close link between the lack of PEPT2 and the expression of these genes.

P63

Norepinephrine transporter knockout-mediated regulation of adrenergic receptor mRNAs in mice brain

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The norepinephrine transporter (NET) is responsible for the rapid removal from the synaptic cleft of released norepinephrine (NE) within the central nervous system (CNS). Inhibition of NET is the primary mechanism of a major group of clinically important antidepressants (such as reboxetine or desipramine). During long-term therapy these drugs lead to alterations in the density of pre- and postsynaptic adrenoceptors, which contributes to the

antidepressant effect. Recently mice with targeted disruption of the gene encoding the NET have been described. However, little is known from these NET knockout (NET-/-) mice about changes in the expression or function of the CNS adrenergic receptors. Therefore we searched for differences (between wild type (WT) and NET-/- mice) in the mRNA expression of the alpha1a-, alpha1b-, alpha1d-, alpha2a-, alpha2b-, alpha2c-, beta1- and beta2-adrenoceptors. We focused on these receptors, since they have been proposed to play a role in the antidepressant effect. In addition, the relative expression in the brain regions of these receptors were determined in WT mice. By quantitative RT real-time-PCR using specific primers for the receptor genes and for certain housekeeping genes (GAPDH, ß-Actin and HRPT1) mRNA expression levels in cortex, cerebellum, brainstem, hippocampus, hypothalamus, striatum and olfactory bulb and from whole brain were determined in relation to the expression of the housekeeping genes. In the NET-/- mice, we observed a specific upregulation of the mRNAs encoding the alpha1b-, alpha2a- and alpha2c-receptor (4-fold, 3.5-fold and 5-fold respectively) in the brainstem and of the alpha2c receptor (about 2-fold) in the hippocampus. In the other regions and in the whole brain no significant change of these receptor mRNAs could be detected. The alpha1a-, alpha1d-, alpha2b-, beta1-, and beta2-receptor mRNA densities were not affected by the NETknock-out. Thus, the most impressive effect induced by the NET knock-out was an increase in mRNA expression of the alpha2a- and alpha2c-adrenoceptors in the brainstem.

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P64

Application of real-time PCR for quantitative determination of transgene copy number in transformed CHO cell lines

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Post-translational modifications of certain glycoproteins used as recombinant drugs need mammalian host cells cells for successful production. Chinese Hamster Ovary cells (CHO cells) have often been used for this purpose, having a number of favorable attributes such as known selection markers, low protein secretion, good glycosilation patterns of glycoproteins and substantial knowledge about long-term culture systems and potential large scale cultivation exists. An expression vector containing cDNA of our glycoprotein was transfected into CHO cells. For the molecular characterization of a transgene-cell line the number of construct insertions has to be determined. We have developed a real-time PCR quantification for this purpose. Two different amplicons were constructed for the 5'-3' exonuclease real-time PCR assay. One in an endogenous single copy gene in hamster genome and the other in the gene coding our glycoprotein. Knowing the size of hamster genome and of the plasmid we were able to prepare our own reference materials containing known ratio between plasmid DNA and hamster genome. Firstly, the concentration of the DNA from non-transformed CHO cells and of the linearised plasmid DNA containing the glycoprotein cDNA was accurately determined spectrophotometrically and by real-time PCR. Than the reference material was prepared by mixing certain amounts of non-transformed CHO DNA with the plasmid DNA (containing the insert) to obtain DNA containing 10, 5, 2 and 1 trangene per genome. Absolute quantification with real-time PCR was used to verify the applicability of prepared reference materials for the determination of transgene copy number in transformed CHO cell lines. This robustness and accuracy of the developed method is now being validated.

P66

Development of real-time RT-PCR assays for relative gene expression estimation of equine cytokines

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Introduction of real-time PCR technology has significantly improved and simplified the quantification of nucleic acids. For example, real-time RT-PCR assays have been widely used to estimate the expression level of genes of interest. The aim of this study was to characterize an early immune response after vaccination of horses with inactivated virus strains. Samples were taken at several time points before and after vaccination in a longitudinal comparative study.
In addition to clinical examination and routine haematology, the relative expression levels of three different important equine cytokines (IL-2, IL-4 and TNFalpa) were estimated using real-time RT-PCR assays. In order to minimize the influence of variations in the expression level of house-keeping genes, three different candidate house-keeping genes (equine glyceraldehydes-3-phosphate dehydrogenase, beta-actin and beta-2 microglobulin) were used to calculate the relative expression levels (ddCt-method).

P67

Modular concept of a real time PCR-assay for determination of the quantity and quality of mitochondrial and nuclear DNA

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Successful DNA-profiling of forensic samples is largely dependent on the quality and the amount of DNA that is recovered from the sample in question. A basic requirement for an optimal result, e.g. a well balanced and complete STR profile, is that the input of template DNA to the amplification reaction mixture lies within the recommended range of the PCR-application. Adding amounts of DNA outside this range will adversely effect proper profiling and can result e.g. in locus imbalance, artifact bands, allelic or locus dropout, or stutter false alleles. Accurate and reliable DNA quantitation is therefore a critical step in the process of successful DNA analysis. But also PCR-inhibitors co-extracted with the sample or degradation of the DNA can negatively impact typing results and lead to the same phenomenons as described above. A quantitation system that additionally could provide information about the presence of inhibitors and the fragmentation grade of the DNA will be of great value not only for predicting PCR-success, but also for the interpretation of the STR-results.

For reliable and sensitive DNA quantitation the application of real-time PCR (rtPCR) is described. We modified a published rtPCR assay - which allows for the combined analysis of nuclear and mitochondrial DNA (mtDNA) - by introducing (1) an internal positive control (IPC, for both nuclear and mtDNA) for monitoring PCR-inhibition and (2) different amplicon fragment lengths for the determination of the degradation state of the DNA.
The IPC's were constructed by site directed PCR-mutagenesis by overlap extension (SOE) of the wild-type mtDNA and nuclear RB1 target in a way that the modified template will not be amplified with the conventional primers, whereas the modified primers would only amplify the SOE-product. The advantages of this procedure are that no additional probes are required, minimizing the costs of the assay. Furthermore fluorescent probes with a Minor Groove Binder (MGB) are used, as they result in greater apparent PCR efficiencies for the amplification of longer fragments compared to standard TaqMan probes. The quantitation system is accomplished as a modular concept, which allows for the combined determination of the above mentioned features (quantitation of nuclear- and mtDNA/ quantitation of nuclear- or mtDNA and detection of PCR-inhibitors/ quantitation of nuclear-DNA and detection of fragmentation) depending on the situation.

P68
The Profiling of Estrogen-Response Elements in Estrogen Target Genes of MCF7 Breast Tumor Cells

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The specific nuclear actions of estrogen are determined by the characteristics of target gene promoter that modulate the final transcriptional response to the complexes of estrogen and estrogen receptors. The estrogen-estrogen receptor complexes bind to the specific sequences of DNA called Estrogen-response Elements (ERE). The exact mechanism of the nuclear translocation of estrogen-estrogen receptor complex remains unknown. Thus, we aimed to investigate the profiles of EREs in the regulatory regions of estrogen target genes in MCF7 breast tumor cells. The MCF7 cells are treated with Estrodiol for 45 minutes followed by crosslinking with formaldehyde. The cells were lysed and sonicated into smaller fragments. The protein of interested was immunoprecipitated by ER and GST polyclonal antibody respectively. Quantitative real-time PCR was performed with SYBR Green I chemistry using LightCycler® to investigate the enrichment profiles of estrogen target genes. Our results demonstrated enrichments in known and novel estrogen target genes. The variant sequences of ERE showed in these genes bind to estrogen receptor with different affinity and induce the transcriptional activity. We showed that estrogenic activities are mediated by the affinity of estrogen receptor in modulating the transcriptional response. Our results have provided better understanding in the mechanism of estrogen receptor and contributed in targeting the particular actions of estrogen with increasing specificity and therefore with more benefits and fewer unwanted side effects.

P69
USE OF REAL-TIME RT-PCR TO COMPARRE GENE EXPRESSION IN MALE AND FEMALE *Brugia malayi*| ADULT WORMS

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Real-time quantitative reverse transcription polymerase chain reaction (RT-PCR) is a powerful method for measurement of gene expression in the study of diverse biological processes. Improved understanding of the biology of reproduction in filarial worms may lead to identification of new targets for drugs or vaccines. Candidate gender-linked genes were selected from genes identified in prior studies by conventional RT-PCR or by an electronic search of the |B. malayi| EST database for clusters with possible gender-specific expression. Expression of candidate genes in male and female worms was compared by real-time RT-PCR with sequence specific primers. Double stranded PCR product was measured by SYBR Green fluorescence; melting curves and agarose gel electrophoresis were used to verify the specificity of results. Gene expression results were normalized with internal controls (genes that are equally expressed in male and female worms such as beta actin 2, histone H3, and NADH dehydrogenase subunit 1). Relative gene expression ratios in male and female worms were quantified by the comparative C_T method. Thirteen of 20 candidate genes were verified to have gender-biased expression with ratios between 4 and >1,000. These included some well-known genes such as major sperm protein (MSP, ratio 724) and fatty-acid binding protein (BmFABP-1, ratio 5) and others not previously studied. Interestingly, no gene exhibited absolute gender-specific expression. These results show that real-time RT-PCR is a powerful method for studying gene expression in *B. malayi*.

P70
Beneficial applications of cell line and primary cell Q-PCR data.

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Analysis of mRNAs for specific genes or entire gene families in mammalian cell lines and primary cells can be highly beneficial for a number of cell-based applications. Examples include checking potential cell hosts for null mRNA backgrounds prior to transfecting genes, identifying cell lines producing mRNA for required accessory proteins when transfecting genes, selecting a cell line with appropriate mRNA level for gene knockdown studies, identifying cell lines with appropriate endogenous mRNA levels for drug target validation assays and also for selecting cell lines producing specific mRNAs for specificity checks and characterisation of antibodies. Q-PCR is an ideal technology for this analysis due to it's sensitivity, specificity and the ability to measure appreciable numbers of gene mRNA levels rapidly.
This poster will describe an initiative to collect all regularly used human cell lines and primary cells from our laboratories and determine the relative levels of mRNA species in 150 human cell lines and primary cells. Data will be presented showing how prior knowledge from these

data can influence subsequent cell-based assay design and improve the chance of experimental success, as well as highlighting appropriate cells for antibody reagent validation and trouble-shooting.

P71
Innate Immune Receptors of the TLR Family in Human Skin Disease: Profiling of Functional Gene Expression in Epidermal Keratinocytes

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The number of individuals suffering from allergic airway and skin diseases such as atopic dermatitis, allergic rhinitis or asthma has increased significantly over the past two decades. The human genetic background together with environmental factors is responsible for the outbreak of these diseases. Amongst the various hypotheses to explain this dramatic increase in allergic diseases, the “hygiene hypothesis” has recently drawn considerable attention: a lack of appropriate microbial stimuli for the immune system during childhood is regarded as an important trigger for the development of atopic allergies. To investigate relevant genes which are associated with allergic skin diseases and to elucidate their interaction with environmental factors, gene expression profiling utilizing real-time PCR and chip microarray technology represent important and promising methods. Recently a new family of microbial pattern recognition receptors, the so called Toll-like receptors (TLR), was discovered. TLRs play an important role in innate immunity. They recognize defined molecules of exogenous microorganisms and promote an adaptive immune response towards them. As skin represents the first defence barrier against invading pathogens and keratinocytes compose the major cell component in human epidermis, assessment of their TLR expression and function can reveal new aspects of the role of keratinocytes in allergy and immunity. In order to do this, constitutive expression of TLR in keratinocytes was investigated with quantitative real-time PCR. Input of cDNA was normalized using the level of GAPDH as internal standard (housekeeping gene) and final quantification was carried out by using related standard curves. Several TLRs showed constitutive expression. We focused on TLR3 which recognizes double stranded viral RNA and performed stimulation experiments with the defined TLR3 ligand poly (I:C) over a time course of 0, 2, 8 and 24 h. Changes in mRNA expression were analysed with Affymetrix chip microarrays and quantitative real-time PCR. The relevance of the findings was confirmed by measuring the protein levels of interleukin-8, a marker of functional TLR3-dependent keratinocyte activation, in cell culture supernatants. To conclude, for the activation-dependent analysis of TLR genes in human keratinocytes real-time PCR was more sensitive than chip microarrays. However, both methods together with functional protein assays are required to define a functional TLR expression and response profile of epidermal skin keratinocytes with relevance for allergic skin diseases.

P72
Quantitative mRNA expression of the IGF-system members during induced luteolysis in the bovine.

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Insulin-like growth factors (IGF I and IGF II), their binding proteins (IGFBP 1-6), IGF-receptor type 1 (IGF-R1) and growth hormone receptor (GH-R) were shown to be differentially regulated during bovine oestrous cycle and pregnancy. We investigated the mRNA expression of these factors during PGF2□-induced luteolysis: Cows in the mid-luteal phase (days 8-12) were injected with the PGF2α-analogue Cloprostenol, and CL were collected by transvaginal ovariectomy at 2, 4, 12, 48 and 64 h after PGF2α-injection. Real-time RT-PCR (LightCycler) using SYBR Green I detection was applied to determine the detailed mRNA expression pattern. Ubiquitin (UBQ) was used as an internal control and for normalisation. The acquired expression data were analysed with the Relative Expression Software Tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. There were no significant changes in IGF I mRNA expression. GH-R, IGFR-1, IGF II, IGFBP-3 and IGFBP-4 mRNA expression was significantly down-regulated in certain stages. IGFBP-2 and IGFBP–6 mRNA was up-regulated ($P < 0.05$) 64 h after induced luteolysis. IGFBP-5 mRNA was up-regulated already at 12 h after PGF2α ($P < 0.05$) with a maximum of an 11-fold increase at 64 h ($P < 0.01$). IGFBP-1 mRNA was significantly up-regulated at 2 h and maximal at 4 h with a 34-fold increase ($P < 0.001$). This study demonstrates that the members of the IGF-system were differentially expressed and may have an important influence on PGF2□induced luteolysis in the bovine corpus luteum.

P73
Abundance of mRNA coding for components of the somatotropic axis in different layers of the jejunum and ileum of neonatal calves

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In calves, the growth hormone (GH)-insulin-like growth factor (IGF) axis and insulin receptors are variably expressed among the different gastrointestinal sites, and are therefore thought to exert site-specific functions. We have examined the levels of mRNA of IGF-1, IGF-2, of receptors for IGF-1 and IGF-2 (IGF-1R, IGF-2R), GH (GHR) and insulin (InsR) and of IGF binding proteins (IGFBP-2 and -3) in different layers of jejunum (villus tips; crypts) and ileum (villus tips; crypts; Peyer patches, PP) by real-time PCR.
Calves (Red Holstein x Simmental, Holstein Friesian), n=9, were colostrum-fed and euthanized on d 5 of life and intestine was quickly removed and flushed with 154 mM NaCl. Rectangular pieces (5x5 mm^2) of jejunal and ileal walls were cut, placed on glass slides, covered with supporting medium (O.C.T. compound, Tissue-Tek®,

Zoeterwoude, Netherlands) and then shock-frozen in liquid nitrogen. The frozen pieces of jejunum and ileum were cut horizontally at –20°C into slices of 10µm depth using a cryotome. Fifteen consecutive and morphologically similar slices were collected as a fraction for the villus, crypt and PP layers, resp. The first and the last slices were stained with haematoxylin-eosin and evaluated microscopically to confirm homology of the fraction. The slice before the last cut was used to assay for the presence of 5'-bromo-2-deoxyuridine (BrdU) that labels proliferating cells. Total RNA was extracted from the combined 12 slices of each fraction using Trizol (Gibco) and diluted to 100 ng RNA/µl. Then one µg of total RNA was reverse transcribed into cDNA. Twenty five ng of cDNA were then used to measure specific mRNA using external DNA dilution curves for each parameter of the somatotropic axis. In addition, to characterize the fractions, mRNA levels of lactase - which served as a brush border marker - was also measured relative to housekeeping genes (hkg). For this purpose the crossing points (CP) were recorded. Based on the hypothesis that hkg are constantly expressed, the mean CP value of glyceraldehyde phosphate hydrogenase (GAPDH), ubiquitin, 18S and β-actin was calculated. The Δ1CP of all hkg, which is the difference of CP of each layer from mean CP of all tissues, was used to correct lactase CP values (as $\Delta 2CP = CP_{lactase} \pm \Delta 1CP$) to get virtually constant hkg gene expression. The corrected lactase expression was calculated as $2^{(-\Delta 2CP)}x100$. Data were analyzed using the SAS program package. Differences between intestinal layers were tested for significance with Mann-Whitney test (jejunum) and Kruskall-Wallis test (ileum).

The BrdU positive cells were found in crypts and PP. Relative lactase mRNA levels in jejunum and ileum were maximal in villus tip fractions (94 and 69%, resp.) and minimal in crypt fractions (6 and 31%, resp.). In jejunum, amounts of total RNA of the villus fraction were 2.5 times lower ($P < 0.05$) than in the crypt fraction, mRNA levels of IGF-1, IGF-2, IGF-1R, IGF-2R, InsR, GHR, IGFBP-2 and IGFBP-3 were higher ($P < 0.05$) in the villus fraction than in the crypt fraction. In ileum, amounts of total RNA in the villus fraction were 3 and 4 times lower, resp., than in crypt and PP fractions, mRNA levels of IGF-1 were higher ($P < 0.05$) in PP than in villi and crypts, and mRNA levels of GHR were higher ($P < 0.1$) in crypts than PP.

In conclusion, members of the somatotropic axis are variably expressed in different jejunal and ileal layers of neonatal calves, pointing on their different importance at different sites of villus-crypt axis and in the submucosa.

P74

Investigations of a unique line of iron-resistant rat cardiac myoblasts using quantitative RT-PCR

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Chronic iron overload is associated with increased oxidative damage, inflammation and fibrosis in tissues such as heart, liver and pancreas. In particular, iron accumulation in heart tissue may result in cardiac dysfunction and failure. We employed a myoblast cell line, H9c2, originally derived from rat embryonic heart tissue, as a model for acute and chronic iron overload to study iron-induced oxidative stress responses and resistance mechanisms. Fluorescent probes were used for detection of reactive oxygen species (ROS) production, cytotoxicity and mitochondrial function. Mitochondrial DNA damage, DNA copy number, and alterations of expression profiles of certain stress genes were quantified by real time PCR. Unexpectedly, we found that, after prolonged exposure (>6 months) to iron (200 µM) in complete culture media, some of these cells became highly resistance to iron induced cytotoxicity: the acute LD_{50} for iron was 2-3 fold higher than for native H9c2 cells. The mitochondria of these iron-resistant cells (whether grown in the presence or absence of iron) produce less ROS and are resistant to mitochondrial DNA damage caused by exogenous H_2O_2. These iron resistant cells also display cross-resistance to hyperoxia (culture under 80-95% oxygen), hypoxia (1% oxygen), the redox cycling drug, menadione, and TNF-alpha induced apoptosis. Of importance, this characteristic of cross-resistance is retained even after long-term culture of these cells in the absence of iron. Analysis of the activities of several mitochondrial respiratory chain enzymes (complexes I, III and IV) and anti-oxidant enzymes (SOD, catalase, and GSH peroxidase) reveals no significant differences. Likewise, microarray and RT-PCR analyses indicate that the mRNA expression profiles of major iron handling (involved in iron uptake, transport and storage), anti-oxidant and metabolic proteins are similar to wild type. However, there is a 2-5 fold increase in the expression of insulin-like growth factor II (IGF-II), an anti-apoptotic and tumor-associated autocrine growth factor. In addition, mRNA for cardiac adriamycin responsive protein (CARP), a transcription factor, is significantly reduced under iron-, hypoxia- and hyperoxia-induced stress. RT-PCR confirms IGF-II over-expression and CARP reduction, one or both of which may be associated with the unique iron tolerance of these cells.

P75

Analysis of Substance P receptor mRNA in periosteum and capsular tissue of adjuvant arthritic rat using hemi-nested RT-PCR

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Introduction:

Expression studies have shown uneven distribution of Substance P receptor (SPR) mRNA throughout the rat central nervous system. The highest level of SPR mRNA was reported in urinary bladder whereas in other tissues it normally occurs below PCR detectable levels. The mRNA level in the rat joint tissues has not yet been reported in the literature. This is the first report that describes the method for the analysis of SPR mRNA in adjuvant derived arthritic rats.

Material and Method:

Arthritis was induced in adult male Sprague-Dawley rats by intra dermal injection of 50 µl of heat-killed *M. butyricum* in paraffin oil into the base of the tail. Following the development of arthritis, the rats were killed and periosteum and capsular tissue were snapped frozen in liquid nitrogen and stored at –70 °C.

Subsequent to total RNA extraction from tissues, cDNA was synthesized and external PCR was performed in 50 µl volume containing PCR buffer, 1.6 mM $MgCl_2$, 0.2 mM dNTPs, 25 pM of each primer and 2 units of AmpliTaq.

PCR program consisted 40 cycles of 94°C for 40 sec, 58°C for 40 sec and 72°C for 90sec. A second PCR was performed using the same forward primer and an internal reverse primer by exactly following the above conditions. Finally, PCR products were analyzed on an agarose gel and amplicons of 327 bp depicted amplification of SPR mRNA. The sequences of primers designed for SPR mRNA detection are as follows; external forward 5-ATGAG CAAGTCTCTG CCAAACG-3;external reverse 5-CTCGCAGC AACTCCAAGA CCA-3;internal reverse 5-CCACCCGA TACCTCCAGA CACA-3.

Results and Conclusion:

We have developed and tested a hemi-nested RT-PCR assay for the detection of SPR mRNA in periosteum and capsular tissues of adjuvant arthritic rats. Our preliminary data show higher expression of SPR mRNA in arthritic rats compared to normal controls. In future we plan to use this test for further SPR studies in adjuvant arthritis and anti inflammatory rat models.

P77

Inducible and endothelial nitric oxide synthases (NOS) mRNA in the bovine ovary

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Nitric oxide (NO) is a small and permeable molecule, which is able to pass membranes by diffusion. It is known to act as a paracrine mediator during various processes associated with female reproduction. It is generated from L-arginine by nitric oxide synthases (NOS). In the present study, the mRNA expression of the inducible (iNOS) and endothelial (eNOS) NO-synthases were examined in the bovine ovary focusing on the follicular development and the corpus luteum (CL) during the estrous cycle and pregnancy. Additionally, in an *in vitro* approach using isolated granulosa cells (GC) the effects of exogenous applied gonadotrophins LH and FSH were analysed. Bovine ovaries were collected at the local slaughterhouse and grouped depending on the stage of the cycle. A classification of follicles (<0.5; >0.5-5; >5-20; >20-180; >180 ng/ml) was performed according to the estradiol-17β (E) content of follicular fluid (FF). The corresponding size of follicles was 5-7, 8-10, 10-13, 12-14 and >14 mm, respectively. Follicular tissue was separated in theca interna (TI) and GC. CL were assigned to the following stages; Days 1-2, 3-4, 5-7, 8-12, 14-17, >18 of oestrous cycle and of early and late pregnancy (<4 and >4 month). Tissues were dissected in small pieces, snap-frozen in liquid nitrogen and stored at –80°C until use. For *in vitro* investigation, GC were recovered by rinsing antral follicles with PBS. After 1.5 days of preculture, the GC were treated with FSH, LH and FSH+LH, respectively (0.01 IU/mL of bovine FSH and/or LH) for 4 or 24h. Total RNA of both tissues and cells was extracted and reverse transcribed (RT). Resulting cDNAs were amplified by conventional PCR and quantitative real-time PCR (LightCycler®) using specific primers for iNOS and eNOS. During follicular growth the mRNA expression of eNOS did not vary ($p>0.05$) in TI, while iNOS was upregulated ($p<0.05$) in large follicles. In GC, iNOS expression tendentiously increased whereas eNOS decreased with follicular size and increasing content of E2 in the FF. iNOS mRNA concentration in CL tended to increase during the late luteal phase (Days 13-18) and regression (Days >18), but markedly decreased ($p<0.05$) during pregnancy. eNOS showed highest ($p<0.05$) mRNA expression during the early luteal phase compared to the late luteal phase, regression and pregnancy. *In vitro*, iNOS exhibited strongest ($p<0.05$) expression after exposure to LH followed by FSH ($p<0.05$). The *in vitro* experiment support the concept that gonadotrophins act as stimulants for iNOS mRNA expression. In conclusion, distinct expression patterns of iNOS and eNOS suggest them to participate in the final growth of the preovulatory follicle and the CL during different functional stages of the bovine ovary. Local mechanisms regulating both NOS during folliculogenesis and luteal development can be assumed.

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P78

Expression of *E coli* gene transcript at various stages of growth, under the control of stationary phase promoters

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A secretion system based on Kil peptide in *E.coli* has already been worked out and now the study is being carried out for the optimisation of this application under different stationary phase promoters and with different heterologous proteins. Real time RT-PCR presently has been a method of choice to quantify transcripts with high sensitivity and high precision.The same has been applied to quantify the level of kil transcript in different phases of bacterial growth including entry into stationary phase. Since our secretion system uses the stationary phase characteristics, other genes like *rpoS* and *rpoD*, which play an orchestrating role in the transition from exponential to stationary phase, were also quantified. Experiments have revealed that levels of *kil* gene shoots up more than hundred fold during this transition phase. Relative induction of the *rpoS* gene also shows an increasing trend during transition. The application of real time RT-PCR in our studies have shown the usability of the methodology leading to measuring differences in promoter strength and to help understand the machinery during stationary phase.

Poster Session: Food Hygiene & GMO & Agrobiotechnology

P76
Determining the expression level of transgene in transformed plants – Method of choice?

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Plant transformation is an important experimental tool for investigations of various aspects of plant biology. Considerable variation among independent transgenic lines obtained under identical conditions using the same DNA construct is observed. Therefore in study of transgenic plants a molecular characterization of each independent transgenic line is needed and it is essential to determine the level of transgene expression in a high number of obtained lines.
Northern blot analysis, reverse transcription semi-quantitative RT-PCR, which are currently used for this purpose, enable only qualitative or semi-quantitative estimation of transgene mRNA levels. We have established a method, which combines reverse transcription and real-time PCR (TaqMan®) assay to determine the level of mRNA of transgene in transformed potato plants *Solanum tuberosum* cv. Igor containing gene for coat protein of PVY^{NTN} (CP PVY^{NTN}).
In comparison with the other two methods we proved that the newly developed Real-Time PCR assay is more sensitive, has a broader dynamic range of quantitation, is more accurate, and it is favourable for further applications in high-throughput molecular characterisation of transgenic lines.

P79
Real-time PCR: accuracy of GMO quantification

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Labelling of food products containing more than 0.9 percent of GMOs is required according to EU legislation. Real-time PCR using specific primers allows identification of transgenic lines and quantification of GMOs. The quantity of GMOs in the sample is expressed as a quotient of the quantity of transgene and the quantity of species specific gene (for example lectine for soybeans). Quantification of both sequences (species specific and transgene) is determined by absolute quantification where the copy number of species specific gene and of the transgene is compared to a standard material with known GMO concentration. Standard curves for species specific gene and the transgene are constructed by preparing serial dilutions of DNA isolated from standard material.
Parameters affecting Real-time PCR reaction were studied in RoundUp Ready soybean model system on ABI PRISM 7900 HT. The limit of detection and limit of quantification for soybean amplicons and the dynamic range of the measurements were evaluated. Variability within one experiment and repeatability (different days and operators) were compared. Efficiency of PCR reaction for lectin and RoundUp Ready soy specific amplicons was calculated for samples of different food products. Real-time PCR reaction efficiency varied between runs on different days and between operators from 85% to 100% although correlation coefficient value is close to 1. Therefore a standard curve must be made for each separate real-time PCR experiment for each amplicon assayed. The efficiencies of both PCR systems vary in different food matrices. These differences can substantially influence the result. The inclusion of sample dilutions is recommended in any Real-time analysis to avoid erroneous quantification.

P80
Real time RT-PCR as a tool to study differential gene expression in the potato-late blight interaction

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Gene expression study is one of the strategies developed to understand biological processes.
We used such a strategy to study the potato-late blight interaction. Late blight, caused by the fungus-like oomycete *Phytophthora infestans*, is one of the most severe potato diseases worldwide and can destroy a potato field within a few days.
To detect differentially expressed genes during this plant-pathogen interaction, a fluorescent cDNA-AFLP technique was applied on four potato genotype pools, differing in their resistance level to late blight and sampled at four different time points. Different expression profiles have been obtained and a total of 262 transcript-derived fragments have been isolated, cloned and sequenced.
Depending on their homology with known putative functions, we focused on some transcripts. Real time RT-PCR allowed an accurate quantification of the selected transcripts in the different samples. In that way, we confirmed the results obtained by cDNA-AFLP and were able to give more precisions as to the quantitative evolution of the expression profiles. Different statistical approaches of the results will be discussed.

P81
Applications for qPCR in applied plant biotechnology

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When developing new traits for plant breeding using biotechnology it is important to have a fast and early selection of plants fulfilling specified criteria regarding functionality as well as regulatory compliance. A method suitable for the high throughput analysis needed on DNA as well as RNA level is real-time PCR. The application of this technique in the analysis pipeline leads to savings both regarding time and resources.

At many different places along the R & D pipeline qPCR has shown to be a valuable tool, e.g. ideally in primary transformed plants the transgenic nucleic acid sequence should be inserted as a single copy at a single locus in the plant genome, without undesired vector backbone. Unfortunately this is often not the case making high throughput identification and selection systems necessary regarding inserted/not inserted DNA, its copy number as well as transgenic RNA expression levels. In further generations in greenhouse as well as in field, additional analyses are necessary not only for verifications, but also e.g. to document the segregation on transgene zygosity levels/copy numbers, stability of expression etc. Analysis of Adventitious Presence and SNP studies are other parts of Plant Biotechnology where qPCR could be used. "TaqMan assays" have been validated and implemented for several plant species at BASF Plant Science and shown to be a valuable tool to complement traditionally used Southern and northern blotting techniques, facilitating the process and speeding up the R & D pipeline.

P82
Real-Time PCR Systems for *Salmonella* spp. and *Listeria monocytogenes* Detection in Food Products

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iQ-Check™ is a commercially available real-time PCR kit (Bio-Rad Laboratories) that detects *Salmonella* spp. and *Listeria monocytogenes* in food and environmental samples. The assays consist in amplifying and detection of highly conserved genes using fluorescent probes. The iQ-Check test consists of a lysis reagent for DNA-extraction, a ready-to-use PCR-buffer containing specific primers, polymerase, nucleotides and the internal control, the fluorescent probes as well as a negative and positive control.
During the amplification, the fluorescent probes hybridize to the generated amplified products. Fluorescence is monitored directly in the tubes and displayed in real time by the iCycler iQ™ thermal cycler (Bio-Rad).

In the present study we have evaluated the specificity and the sensitivity of the systems.
For internal studies (specificity) with iQ-Check *Salmonella* 72 *Salmonella* isolates were found positive and 51 other bacteria strains (including 15 *Enterobacteriaceae*) were found negative. With iQ-Check *Listeria monocytogenes* 50 different *L. monocytogenes* species were found positive and 39 other bacteria strains (including 16 *Listeria* other than *monocytogenes*) were found negative.

The sensitivity of the methods was tested using pure target DNA in serial dilutions, pure bacterial suspensions and a variety of food samples naturally and artificially contaminated with bacteria. The homogeneous assays for *Salmonella* spp. and *Listeria monocytogenes* have a sensitivity of 5 target gene copies and detect 10^2 CFU/ml of pure bacterial culture.

With both iQ-Check tests 1 to 10 CFU were detected in 25 g of various contaminated samples. These data indicate that iQ-Check is a highly specific and sensitive test.

P83
Quantitative PCR in a high-throughput agricultural biotechnology setting

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Quantitative PCR analysis has proven to be a valuable tool in the process of development of improved maize hybrids. Copy number and zygosity quantification, SNP detection, and relative gene expression results are generated using optimized, simplified procedures and robust robotic systems. An overview of our QPCR and data analysis process is presented, along with future goals and directions for process enhancement.

P84
SOD, CAT and GSH-Px mRNA expression in liver of Atlantic salmon *Salmo salar* exposed to hyperoxic conditions during smoltification

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Real-time PCR has become the new state-of-the-art method for single gene expression analysis. The technology offers high throughput, and combines high sensitivity with reliable real-time fluorescence specificity. A recent study showed that Atlantic salmon *Salmo salar* smolt (start weight 44 g), exposed to supersaturated freshwater (O_2-levels ranging from 110 – 130%, representing values measured under intensive rearing conditions), had a significantly reduced specific growth rates compared to control fish in 90% O_2. In this study, the mRNA levels of three important antioxidant genes; superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px), were quantified with real-time RT-PCR (ABI Prism 7000 Sequence Detection System). Tissue samples of smolt exposed to hyperoxic conditions during the critical smoltification phase were analyzed in order to develop biomarkers for oxidative stress in fish. Total RNA was isolated from liver of 54 fish. Gene specific primers and Taqman probes were designed accordingly. 18S and β–actin were chosen as reference genes, and all samples were run in triplicates. For efficient evaluation and statistical analysis of the data the Microsoft Excel-based software Q-Gene was used to calculate mean normalized expression (MNE). The Q-Gene tool was developed to manage and expedite the entire experimental process of quantitative real-time RT-PCR, and is offered at no cost from the BioTechniques Software Library. The MNE data were then log-transformed, and statistical differences between groups of differently exposed fish evaluated with student t-test with the GraphPad InStat software. This experiment revealed that smolt exposed to the highest levels of superoxygenation conditions had significantly increased liver mRNA levels of SOD (1.5 times increase, $p = 0.044$), CAT (2.7 times increase, $p = 0.002$) and GSH-Px (4.8 times increase, $p = 0.011$), compared to the control fish ($n = 6$). The real-time PCR technology can therefore be of great value in future aquatic biomarker assessments for risk evaluation.

P85

The use of Real-Time PCR for GMO detection: Comparison of commercial and in-house protocols.

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Several Genetically Modified Organisms (GMOs) have been authorised, released into the environment and placed on the market in EC. Community legislation on GMOs has been in place since the early 1990s and throughout the decade, this regulatory framework has been further extended and refined. The Novel Foods Regulation provides for the mandatory labelling of foods and food ingredients which contain or consist of a GMO without prejudice to the other labelling requirements of Community law. Consequently control mechanisms have been introduced. They include also laboratory analysis of GMO content. Real-time PCR is usually used to quantify GMO content. Methods used for legislative implementation should be robust and should produce accurate, precise, reproducible results. Using a set of IMMR standards and analytical samples we compared (1) SybrGreen and Taq Man approaches, (2) TaqMan GMO 35S Soya Detection Kits and transgene-specific protocol (modified protocl according to Vaitilingom et al. J. Agric. Food Chem., 47, 5261-5266), (3) multiplex vs. uniplex and (4) calculations. ABI PRISM 7700 was used. It was apparent, that SYBRGREEN provided results with unsatisfactory specificity and accurancy. Results obtained with 35S Soya Detection Kits (multiplex) were accurate, with required detection and quantitation limit (10 (LOD) resp.50 (LOQ) copies of target sequence). Trasgene-specific procedure generates results with similar parameters using the same analyte (multiplex and uniplex). Uniplex reaction was less accurate in comparison with multiplex. Transgene-specific procedure is more specific in comparison with 35S Soya Detection Kit. Detailed results will be discussed.

P86

PCR Amplification: Is it simple for Wildlife Forensic Samples?

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One of the mandates of the Wildlife Forensic Initiatives undertaken at the Wildlife Institute of India (WII) are to standardize DNA based techniques for identifying species from various parts and products and provide support to enforcement agencies for implementation of *Indian Wildlife (Protection)* Act *1972.* Wildlife Forensic samples of more than 250 meat cases contain quantities of DNA too small for a reliable analysis, as samples are always degraded or putrefied in nature. We have performed PCR on a number of wildlife samples ranging from hair, fresh tissue, cooked meat, blood, bear bile, musk pod, scat, formalin preserved meat and feathers. We used primers for PCR which were complementary to conserved areas of the vertebrate mitochondrial Cytochrome b (Cyt b) gene and yielded a 359 bp fragment. The PCR amplification success rate across DNA extracted by various respective methods was in the order of Qiagen > P/C > Chelex. 50 % DNA samples extracted by P/C and Chelex yielded good quality PCR products. In case of Qiagen kit, 66.6% PCR products were of good to very good quality. We used six restriction endonucleases viz. *Hae III, Alu I, Rsa I, Msp I, Eco RI*, and *Hind* III for sambar (*Cervus unicolor*) and chital (*Axis axis*). The success rate of restriction digestion in relation to various DNA extraction methods were in the order of Qiagen (90%) > Chelex (75%) > P/C(55%). Data indicates that three enzymes viz. *Hae III, Rsa I* and *Msp I* showed restriction sites. It is possible to distinguish between sambar and chital by using *Hae III* and *Rsa I*. It was possible to distinguish mongoose from other studied species using *Hae III* and *Rsa I.* We used 17 samples for RAPD amplification out of which the success rate in amplification using both the primers was only 29 %. While comparing RAPD amplification across three methods used for DNA extraction, the success rate was in the order of Qiagen (75%) > P/C (25%) > Chelex (0%) Data reveals that RAPD techniques may not be much useful for discriminating and identifying species from forensic samples. 47% of the above mentioned biological samples (n=184) resulted only in successful amplification for species identification using techniques like, RAPD, PCR-RFLP or for DNA sequencing. We noticed that the amplified products (n=85) are either good (49%) or faint (42%) or very faint (8%). The reasons being poor quality of samples, lack of proper preservation and PCR inhibitors which requires more PCR optimization for successful amplification.

P87

Expression studies of lipid metabolism genes in Atlantic salmon (*Salmo salar*) – effects of replacing dietary fish oil with rapeseed oil.

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As part of the EU-project RAFOA (Researching Alternatives to Fish Oils in Aquaculture, Q5RS-2000-30058) the aim of the current study was to examine the expression of genes encoding fatty acid binding proteins (liverFABP, lFABP and muscleFABP, mFABP) and Δ5 desaturase in Atlantic salmon post-smolt (0.14 to 2 kg) when fed different dietary oils. Both microarray and quantitative RealTime PCR (RT-PCR) techniques were used to study expression levels of liver genes involved in lipid metabolism after 20 and 42 weeks of feeding. The microarray contained probes mainly from Atlantic salmon spotted on a glass slide. cDNA produced from liver m RNA from fish fed 100% fish oil (FO) or 75% rapeseed oil (RO) for 42 weeks were labelled with Cy3 and Cy5 fluorophores and hybridised to the microarray. The microarray identified only minor up- or down-regulation of different lipid metabolism genes. However, significant up-regulation of desaturase genes was detected in livers from salmon fed 75% RO. By using quantitative RT-PCR the expression of mFABP, lFABP genes was measured in red muscle, white muscle and liver. Further, expression of Δ5 desaturase (gi:18958527) was measured in liver, both by using 18S (gi:18152529) and E1Ø (gi:11596419) as reference genes. No differences were found in lFABP or mFABP expression between the dietary treatments, whereas Δ5 desaturase expression in liver was up-regulated 2.2 fold in fish from the 75% RO group compared to the 100% FO group.

P88

Development of Real-time PCR kit for detection and quantification of genetically modified soybean in food and feeds.

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Necessity of control of genetically modified plants spreading in the food market requires development of analytical methods for detection of genetically modified components in food. In the food-processing industry soybean raw material, including genetically modified soybean, is widely used. Majority of created genetically modified soybean lines have 35S promoter of cauliflower mosaic virus, which may be used as a convenient target for screening and quantification. We have developed Real-time PCR kit for detection and quantification of genetically modified soybean in food and feeds for Rotor Gene instrument (Corbett Research, Australia). Kit includes our own set of reagents for DNA isolation, primers and TaqMan probes for 35S promoter and for genomic soybean DNA detection in one PCR tube. This approach allows to calculate the relative amount of genetically modified soybean DNA from all soybean DNA amount in sample. Our technique of DNA isolation is more simple and rapid in comparison with traditionally used for plant CTAB method. It may be used for any food, feeds and raw material, and allows to receive about 2,5 microgram of soybean DNA (measured by fluorimetry) from 20 mg of soybean flour. Quantitation of relative amount of genetically modified soybean component was performed by regression analysis between Ct of 35S promoter and Ct of soybean genome target value difference and common logarithm of genetically modified soybean amount (in percent) in calibrators. As the calibrators we use DNA samples derived from the certified reference material (Institute of Reference Materials and Measurements [IRMM], Belgium) containing 0,1%, 1% and 5% of Roundup Ready soybean flour in unmodified soybean flour. Analytical characteristics of our kit were determined by testing the standards of certified reference material (IRMM, Belgium) containing 0,1%, 0,5%, 1%, 2%, 5%, 0% and 100% of Roundup Ready soybean flour in unmodified soybean flour, as well as food and raw material samples with genetically modified soybean or with unmodified soybean, which were previously tested with recommended by Lipp M. (1999) method using CTAB DNA isolation. Results have shown, that with the developed kit it is possible to detect the presence of genetically modified soybean in food even if its amount is 0,01 % from unmodified analogue. The limit of detection measured by exhaustive dilution limit was 5 copies of 35S promoter or soybean genomic target in PCR reaction. All samples containing genetically modified soybean gave true-positive results. Samples free of genetically modified soybean components did not give false-positive results (100% specificity). Dynamic range was determined from 0,05% to 50% of Roundup Ready soybean flour in unmodified soybean flour. Evaluated limit of quantification was 0,1% of Roundup Ready soybean flour in non modified soybean flour. Relative amount of modified soybean flour in unmodified soybean flour content was measured in the range of 0,1% to 5% with coefficient of variation after regression analysis of 12% (for IRMM standard 0,1%) and 17% (for IRMM standards 1% and 5%). Furthermore it has been proved that our kit may be used on a wide spectrum of the foodstuff and feeds containing soybean raw material.

P89

Expression of the myosin heavy chain gene (MyHC) in Atlantic salmon (*Salmo salar* L.) fed with graded amounts of solubilised protein

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To be able to link growth and protein synthesis in Atlantic salmon (*Salmo salar* L.) fed with graded amounts of solubilised protein, the mRNA expression of myosin heavy chain (MyHC) was used as a genetic marker. MyHC is present throughout muscle development in fish and are consistent with the hyperplastic growth of fish. MyHC primers and Taqman probes were designed with Assay-by-design and real-time PCR analysis performed with the ABI Prism 7000 SDS from Applied Biosystems. Total RNA was isolated from fast muscle samples (N=33) from salmon fed tree dietary solubilised protein levels (low, medium and high). As references genes 18S and elongation factor 1 alpha were used, and all samples were run in triplicates. For evaluation of the expression data the Microsoft Excel-based software REST® (relative expression software tool) was used for group-wise comparison and statistical analysis. The results showed that a medium level of solubilised dietary protein gave a significantly up-regulation of MyHC in A. salmon, by a factor of 2.6 compared to fish fed lower dietary solubilised protein level. At same time there was a significantly higher MyHC expression (factor 1.3) in fish fed medium solubilised protein level compared to the highest level. The MyHC expression results correlate highly to the specific growth rate of the salmon studied. These results showed that real-time PCR technology can provide new valuable knowledge on physiological regulation in growing fish.

P90

A rapid real-time PCR detection method for specific bacteria

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Along with real-time PCR the monitoring of specific nucleic acid sequences has become much more feasible but still the rapid detection of nucleic acids is often cumbersome due to the time consuming and laborious steps of sample pre-handling. This is why we have developed a rapid quantitative nucleic acid detection method amenable to automation. The monitoring of our model analytes, *Listeria monocytogenes* and *Bacillus subtilis*, is accomplished by using a novel, rapid sample pretreatment method, fast thermal cycling and homogeneous real-time PCR based on environment sensitive lanthanide chelates and time-resolved fluorescence. The detection of *B. subtilis* sub gene was accomplished from both *B. subtilis* cells and endospores. *Listeria monocytogenes* was monitored from spiked milk, cheese and fish samples. The method can

detect as little as 1 CFU from crude sample (confirmed with platings). Using a fast thermocycler, the whole process of sample preparation, target sequence amplification and identification can be completed in less than 60 minutes.

P135
"Real-time Detection and Quantitation of Genetically Modified Soy."

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In this study, real-time quantitative PCR was used to detect and quantify genetically modified soy in soy-containing foods. Two different protocols were developed: the first was a one-color assay that used the double-stranded DNA binding dye SYBR® Green I to detect both the Roundup Ready® sequence and an endogenous reference sequence; the second was a two-color assay that used differentially labeled TaqMan® probes to detect the Cauliflower Mosaic Virus (CaMV) 35S promoter sequence (the promoter used to express the transgene) and an endogenous reference sequence. The amount of genetically modified soy was quantified by interpolation against a standard curve generated from a set of certified reference standards. The methods presented here have several attractive features, including: they require only small amounts of starting material; they are suitable for raw and processed foods; and they are adaptable for other genetically modified crops.

P136

Ligation-dependent probe amplification for the simultaneous event-specific detection and relative quantification of DNA from different genetically modified organisms.

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The application of a ligation-dependent probe amplification (LPA) technique for the simultaneous, event-specific detection and relative quantification of DNA from genetically modified organisms in foods is described. This novel approach uses different pairs of synthetic probes, which hybridize to each of the target sequences. Criteria for the selection of the targeted hybridization sites are (*i*) similarity in thermodynamic properties and (*ii*) nearly constant lengths. After hybridization, the probes are bound by the action of a thermostable DNA ligase. Ligated probes possess identical λ-DNA-derived primer binding sites at their ends, enabling subsequent competitive amplification using one common pair of primers. Labelling one of the primers with fluorescein (FAM) permits the amplification products to be detected *via* capillary electrophoresis. Differentiation of amplicons according to their lengths is achieved by the introduction of arbitrary spacer sequences between hybridization sites and the primer binding sites in the probes, resulting in ligation products exhibiting characteristic lengths for each of the targets. The method was designed to allow the detection of reference genes in the genomes from maize and soya, as well as event-specific regions of the transgenic maize line MON810 and Roundup Ready soya. Specificity, sensitivity and parameters related to the application of this system for quantifications were assayed using mixtures of DNA extracted from certified reference maize and soybean flours. This strategy results in a modular system which can be complemented by further probes to broaden the range of target sequences.

Poster Session: New Methods & Approaches

P91
Nucleic Acids Research Group (NARG) Taqman® primer/probe design study

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The Nucleic Acids Research Group (NARG) conducted an empirical study to define parameters required to design an optimal primer/probe set for a 5'-nuclease (Taqman®) real-time PCR assay. New assay design can be one of the major rate-limiting steps in acquiring data rapidly from genes of interest. Although a large number of pre-made assays can be purchased from commercial vendors, it is much more cost effective to design and purchase your own primer/probe sets when a large number of samples are involved. There are general guidelines available concerning assay design, however, exactly how important each of these parameters is has not been studied in an empirical manner. Further, there may be as yet unknown factors that should be taken into account during assay design. The purpose of this study was to give investigators an opportunity to design what they felt is an optimal primer/probe set for a common transcript and then have them tested empirically for effectiveness. Members of the NARG synthesized the primers/ probe sets and tested them using a plasmid containing the mouse IFN cDNA clone as a template standard. Effectiveness of primer/probe design was judged by PCR efficiency (slope), delta Rn, y-intercept and Ct. The goal was to provide an opportunity for participants to sharpen their skills and/or

learn some new ones while demonstrating the principles of Taqman® primer/probe design.

P92
A new reporter dye for real-time PCR binding in the minor groove

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A new unspecific DNA-binding dye has been developed and evaluated for its use in real-time PCR [1]. The unsymmetrical cyanine dye, called BEBO, binds to the minor groove of the DNA double helix. Upon binding, BEBO fluorescence is increased 200-fold. This is the highest reported for a minor groove binding dye, and allows for sensitive detection of DNA in standard real-time PCR instruments. The binding in the minor groove makes BEBO highly selective for double stranded DNA, which is an advantage when probing formation of double-stranded products in real-time PCR against a background of single-stranded primers. The minor groove selectivity of BEBO was engineered by constructing a crescent shape structure that fits the path of the DNA helix [2].

References:
1. Bengtsson M, Karlsson H J, Westman G, Kubista M. (2003) A new minor groove binding asymmetric cyanine reporter dye for real-time PCR. *Nucleic Acids Research,* 31: e45
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P93
Relative Gene Expression Studies using Multiplex Quantitative PCR on the Bio-Rad iCycler iQ® Real-Time PCR Detection System.

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Real-time PCR is a powerful advancement of the basic PCR technique, in which starting amounts of RNA and DNA can be accurately quantitated with appropriate fluorescent detection strategies and instrumentation. Proper experimental design is essential for studies involving relative or absolute comparisons between two or more genes amplified in the same tube, a technique known as multiplexing. Combining the techniques of reverse transcription PCR (rt-PCR) and multiplex real-time PCR (qPCR) is proving to be one of the most powerful tools in the modern researcher's arsenal to study relative gene expression in many diverse experimental systems. We will present data comparing the relative expression of several genes involved in the polyamine biosynthesis pathway, including ornithine decarboxylase (ODC) and S-adenysyl methionine decarboxylase (SAMDC), in the human thymus and prostate. In addition, guidelines for optimization of rt-PCR and multiplex qPCR experiments quantitating up to four gene targets in a single reaction will be discussed.

P94
Unique formulations for quantitative PCR and RT-PCR applications

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Sigma-Aldrich has developed quantitative PCR and RT-PCR products to meet a wide range of researcher needs. These products are designed to work with different detection chemistries and instruments for quantitative PCR and RT-PCR, as each requires different product formulations. JumpStart™ Taq ReadyMix™ for quantitative PCR is ideal for high throughput and offers maximum flexibility in detection method since no detection chemistry has been incorporated into the formulation. For contamination control concerns, JumpStart™ Taq ReadyMix™ with dUTP incorporates dUTP in the place of TTP and may be used on both capillary and noncapillary real-time thermal cyclers. Universal detection chemistry is incorporated into several SYBR® Green I dye-containing products which have recently become available. Sigma-Aldrich offers a specially formulated SYBR Green quantitative PCR ReadyMix for use on capillary-based platforms, such as the Roche LightCycler™, while a second SYBR Green quantitative PCR ReadyMix is formulated for plate/tube real-time thermal cyclers. Finally, Sigma-Aldrich's SYBR Green RT-PCR kit is suitable for gene expression studies using either real-time instrument platform. Data is presented showing the sensitivity, application and capabilities of this new series of Sigma-Aldrich products.

P95
Multiplex qPCR LightCycler Analysis

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Multiplex PCR employs different primer pairs in the same amplification reaction. HybProbes carrying different FRET pairs with different emission spectra allow real time detection of up to 4 targets in parallel using LightCycler 2.0. These hybridization probes afford a level of discrimination impossible to obtain with SYBR Green, since they will only hybridize to true targets in a PCR and not to primer-dimers or other spurious products. The feasibility of Multiplex PCR using HybProbes is shown for mutation detection, determination of expression levels and relative quantification of targets.

P96
Comparison of qPCR with FISH-based enumeration of undefined starter cultures

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In many dairies undefined mixtures of lactic acid bacteria are the most extensively used starter cultures, particularly in European countries. While undefined cultures offer significant advantages in terms of their technological properties such as aroma formation and phage resistance, the flora composition is frequently not well characterized. Conventional plate counting based on selective media has

been the chosen method in most microbiological laboratories to analyse the composition of these cultures. One of the major drawbacks of cultivation-based assays is its rather high time-consumption of about 5 days. In this study, quantitative PCR was used to enumerate important lactic acid bacteria of undefined starter cultures. Both, Molecular Beacons and Taqman probes were evaluated to detect specific PCR amplicons. A multiplex assay was developed to quantify subpopulations relative to total bacterial DNA. The technique was compared with whole-cell fluorescence in situ hybridization (FISH) and conventional cultivation-based enumeration.

P97
Dynamical simulation of a typical real-time PCR system for quantitative DNA detection

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Recent progress in the development of PCR assay techniques combined with new fluorescent methods have led to the introduction of quantitative real-time PCR assays of high sensitivity and precision.Various mathematical methods are used to analyze the time course of the typical gene amplification process.The calibration functions which are normally applied for the quantitative DNA detection are based on semi-empirical exponential or sigmoid „models“, where the system parameters are fitted by linear or non-linear regression of the experimental observation .They do not have a direct physico-chemical significance in the complex interaction of the system variables. Only for limited time-intervals , PCR „efficiency“ can be interpreted in kinetic terms , so that the influence of reaction conditions like cycle interval time, reagent concentration (or activity) and initial DNA sample concentration on the performance of the amplification process can not easily predicted.
We propose a full dynamical simulation of a simplified reaction system using seven system variables (DNA target sequence as monomer and dimer ,primers, DNA-primer adduct, nucleotides and DNA polymerase) together with eight kinetic constants as a systemtheoretical PCR model. The mathematical formulation of the amplification process of a typical polymerase chain reactor can demonstrate the influence of the system parameters under real physico-chemical conditions. Simulation shows to what range the semi-empirical functions normally used in PCR assays are really valid. Our results suggest that a systemtheoretical approach of PCR kinetics can be a very useful tool in PCR assay optimization and validation.

P98
RealMaster Probe Kits – A Novel System for Quantitative PCR with Target-specific Probes

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Real-time PCR uses fluorescent reporter molecules to quantify nucleic acids for assays such as gene expression quantification or GMO testing. These fluorescent reporters can be non-specific intercalation dyes or specifically designed fluorescent oligonucleotide probes. Based on the nature of these reporter molecules, specific reaction chemistries are required.
Eppendorf has developed a quantitative PCR reaction chemistry uniquely optimized for probe-based assays that addresses the exonuclease-dependent signal release reaction. While common quantitative PCR reagent kits are focused primarily on the optimization of the polymerase-driven amplification, or the activation of the inactivated hot-start polymerase, the RealMaster system also targets the signal release by nucleolytic probe degradation. As a result of this optimization, more signal can be generated with the same amount of target DNA.
The RealMaster probe series features the patent-pending Eppendorf HotMaster *Taq* DNA Polymerase, which prevents non-specific product amplification, increases sensitivity, and facilitates room-temperature reaction set-up. The proprietary inhibitor reversibly binds to the polymerase in a temperature-dependent manner so that no lengthy heat-activation is required. Furthermore, the inhibitor is not heat-denatured and provides this control throughout the reaction.
The RealMaster buffer chemistry continually adjusts the concentration of free magnesium, and features a pre-optimized combination of natural and chemically modified dNTPs, enhancers and stabilizers. These components work synergistically to provide outstanding reproducibility, suppress primer-dimer formation, improve signal-to-noise ratio, increase sensitivity and retain peak performance through numerous freeze-thaw cycles.
Two real-time PCR platforms, the ABI 7000 SDS and the Bio-Rad iCycler iQ, are used to amplify two genomic targets to show the increased signal-to-noise ratio achieved with a multi-plex reaction. These same targets show the improvement in reproducibility, whereas the increase in sensitivity is shown using a single-copy genomic target. Finally, the ability of this system to endure multiple freeze-thaw cycles and still retain peak performance is shown.

P99
Mass spectrometric gene expression analysis – combining competitive PCR and MALDI TOF mass spectrometry.

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Here we describe a transcriptional profiling platform that combines competitive PCR and quantitative MALDI-TOF mass spectrometry. cDNA is mixed with a synthetic DNA molecule (the competitor or internal standard). The internal standard matches the sequence of the targeted cDNA region in all positions but one single base. The two distinct sequence species mimic the situation of two different alleles allowing for the use of quantitative MALDI-TOF MS analysis based on primer extension products.
Quantification of the amount of cDNA present in the reaction is achieved by analyzing the ratios of the peak areas. A ratio of one-to-one represents equal amounts of cDNA and competitor template. Serial dilutions of internal standards can be used to gain a broad dynamic range of up to 6 logarithmic units. The quantification is independent of the PCR cycle number and the reaction is highly sensitive (amplification of down to 5 copies of DNA has been obtained). Multiplexing is possible (5-plexes have been achieved with good accuracy) and relative as well as standard curve quantification can be performed.

P100
Development of methods for detection and quantification of mRNA transcripts from single cells

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Cells are in many aspects unique in their characteristics, even in a seemingly homogenous culture or tissue. Traditionally, gene expression analysis look at the average transcript levels of the prepared cells. However, it is often interesting to distinguish a wide-spread response in the whole population from a rare change in expression in a few cells. The natural quantitative variation in individual cells is virtually an undiscovered area of research due to technical difficulties.

We have developed new methods to improve the fidelity and robustness of single cell gene expression analysis using reverse transcription real-time PCR. Special attention was paid to adsorption phenomena, dilution effects, and inhibition of PCR by RT reaction mixture. Four genes were studied and their expressions were compared on the level of individual cells. To increase the accuracy in the quantification of expression levels in individual cells, where the amount of material is very small, a new quantification strategy named *in-situ by addition* is proposed and evaluated.

P101
The Kinetic and Mathematical Model of PCR Amplification Experiment

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The PCR technique has been set up for nearly twenty years and is becoming more and more ripe. But because of the multiple influencing factors and complicated reaction procedures, no mathematical method that can describe the PCR reaction has been given. On the basis of its elementary principle, we suggested a kinetic equation to describe the reaction procedure, $Wamp=[Ntarg\times(1+P)^{nl}+0.5\times Cenz\times U\times P\times Ceactive\times(n-nl)-Ntarg\times(1+n\times P)]\times Cu\times M$, $Famp=[Ntarg\times(1+P)^{nl}+0.5\times Cenz\times U\times P\times Ceactive\times(n-nl)-Ntarg\times(1+n\times P)]\times Fc$ This equation can describe correctly the accumulation rule of PCR product and thus build up the kinetic-mathematical model of PCR reaction. The predicted C_T value of PE 7700 by the kinetic-mathematical model was in accordance with the real value detected by the machine. This kinetic-mathematical model accompanied by proper detecting equipment and computer could make an automatic PCR instrument, which would produce much better result. A laboratory can predict the amount of PCR product by this model and provide accurate information for further handling of PCR product according to its own condition. In this model, the molecular basis that PCR reaction is doomed to change from exponential amplification to linear amplification had been clarified.

Ntarg **primary template**
Ptime **predenaturation time**
P **mean amplification efficiency**
N **cycle**
n1 **transformation parameter**
Cenz **enzyme molecular constant 3.2×10^{10}**
M **molecular weight**
Time **denaturation time**
U **enzyme unit in the system**
Temp **denaturation temperature**
Cu **transformation coefficient (1.66×10^{-18})**
Ceactiv **enzyme activity $1-0.5\times(n\times Time+Ptime)/e^{(a+b\times Temp)}$**
Famp **fluorescence**
Fc **molecular fluorescence**

Key words: PCR PCR kinetic kinetic-mathematical model

P103
Evaluation of the performance of LNA and MGB probes in 50-nuclease PCR assays.

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The aim of this study was to evaluate the use of Locked Nucleic Acids (LNA) probes in 50-nuclease PCR, by comparison with Minor Groove Binder (MGB) probes routinely practiced in laboratories on ABI Prism 7700. The comparison was made using a collection of Staphylococcus aureus strains that have already been characterized by MGB 50-nuclease PCR assays in a previous study [Mol Cell Probes, submitted for publication]. The sensitivity and specificity of 50-nuclease PCR assays targeting the Staphylococcal enterotoxin genes sea to see were compared and showed that the LNA and MGB methods were equivalent. In conclusion, the LNA 50-nuclease PCR assays developed in this work provide a specific and sensitive alternative to the well-established MGB 50-nuclease PCR assays used for the rapid detection of bacterial pathogens genes on ABI Prism 7700.

P104
Real-time immuno-PCR

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Real-time PCR as a tool for DNA quantification or gene expression analysis is now used all over the world. As the technique and the real-time PCR instruments get wider spread, new methods using the technique are developed. One relatively new method uses real-time PCR for quantification of specific proteins. The method is called real-time immuno-PCR. To perform the assay the protein analyte is identified and captured by specific antibodies in a PCR-tube. To quantify the analyte a second antibody, coupled to a DNA-label, is allowed to bind to the analyte, and the DNA-label is then quantified with real-time PCR.

The amount of quantified DNA corresponds to the amount of the protein present in the sample. We have developed a sensitive assay for the quantification of Prostate Specific Antigen, PSA, which is a serum marker for disease in the prostate. In our development we have investigated several different factors that affect the sensitivity and specificity of the assay. One very important step is to block the PCR-tubes to prevent the DNA-label to bind unspecifically to the tubes and give rise to background signal. We have also tested different types of strategies for setting up the assay and different types of PCR tubes.

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qPCR data processing: comparison of relative quantification methods.

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Introduction
PCR is a powerful and sensitive tool for biomedical research and one of the most widely used techniques in mRNA analysis[1].
The accuracy of the measurements obtained by these reactions is dependent on different factors, such as the sample preparation method, the relative efficiency of the PCR amplification and the reliability of the detection assay[2].
The first aim of this work was to compare chemistries from different suppliers for the Ct values and efficiencies obtained with the same samples. The second aim was to compare different ways to treat the data obtained.

Material and methods
Total RNA isolation: bone cells lysates were loaded on Nucleospin[R] RNA II columns (Macherey-Nagel, Düren, Germany), according to the protocol furnished. RNA was eluted in 40□l of RNase-free water and stored at –80°C.
First Strand Synthesis: for each sample, 5µl of total RNA were reverse-transcribed using the Taqman Universal PCR reagents (Applied Biosystems, Foster City, CA) or the Stratascript enzyme (Stratagene, La Jolla, CA) with random hexamers. Reaction volumes were fixed at 50□l. The thermal cycler PCT-0100 (MJ Research, Waltham, MA) was programmed as following : 25°C 10min/ 48°C 30min/ 95°C 5min for the AB chemistry and 42°C 60min/ 70°C 15min for the Stratagene's one.
Real Time PCR: specific primers for □-actin and osteoprotegerin were designed with the Primer Express[R] software (Applied Biosystems, Foster City, CA) and purchased from Integrated DNA Technologies (Coralville, IA). An additional sequence of 18bp was added to the 5' end of forward primers in order to use the Amplifluor[R] Uniprimer[R] technology based on the hairpin configuration (Intergen Discovery Products, Purchase, NY).
Specific primers and probes for the same two genes of interest were ordered using the Assay-On-Demand service (Applied Biosystems, Foster City, CA).
PCR reactions were done in duplicates and monitored with the ABI Prism 7700 and all Ct were obtained with the same fixed threshold.
Data processing: in order to quantify the relative expression of the osteoprotegerin gene, the following methods were compared:
- with a PCR efficiency assumed to be optimal (E = 2) and using the □□Ct formulas[3]
- with a PCR efficiency calculated using the LingRegPCR[4] software (mean of 6 wells) and the Ct values (mean of duplicates)
- with a PCR efficiency calculated using the LingRegPCR[4] software (mean of duplicates) and the Ct values (mean of duplicates)
- with a calculated efficiency using the LingRegPCR software and the individual Ct value for each reaction well.
- with the N_0 value obtained with LingRegPCR.

Results and Discussion
The reverse transcription performed with the Taqman Universal PCR reagents followed by the Assay-On-Demand PCR gave the lowest Ct values (Table 1).

	β-actin	OPG	
	Ct (mean)	Ct (mean)	Ct (stdev)
RT AB + AOD	23.21	28.9	0.21
RT Strat + AOD	24.1	30.05	0.15
RT AB + Uniprimer	25.86	33.87	0.19
RT Strat + Uniprimer	32.16	40.46	0.25

Table. 1: Ct values obtained with different chemistries.

The reverse transcription using AB's transcriptase enzyme followed by the Assay-On-Demand PCR gave the best efficiencies values and the smallest standard deviations (Table 2).

	UniPrimer		
	E (mean)	E (stdev)	R^2 (mean)
β-actin			
RT Strat	**1.749**	0.105	0.998
RT AB	1.623	0.032	0.999
OPG			
RT Strat	**1.740**	0.071	0.998
RT AB	1.718	0.035	0.999

	Assay on Demand		
	E (mean)	E (stdev)	R^2 (mean)
β-actin			
RT Strat	1.968	0.144	0.997
RT AB	**1.915**	0.079	1
OPG			
RT Strat	2.010	0.052	0.996
RT AB	**2.054**	0.151	0.999

Table 2: PCR efficiencies obtained with different reverse-transcription and PCR reagents for the same samples and the same genes targeted.

Using the ΔΔCt calculations or the PCR efficiency calculated using the LingRegPCR software (mean of 6 wells) and the Ct values (mean of duplicates), results are similar between Uniprimer and Assay-On-Demand. The three other methods of analysis gave results which were inconsistent. In this study, we showed that the observed PCR efficiencies could guide the choice of reagents for the reverse transcription and the PCR. Models used for analysis should be choosen with care in order to avoid misinterpretation.

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P106

A Novel Method for Molecular Haplotyping Combining An Improved AS-PCR Technique with Base Specific Cleavage of Nucleic Acids Analyzed By Mass Spectrometry

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Haplotypes, the units of inheritance, can provide additional power in detecting genes involved in common diseases such as cancer. Here we describe a novel approach that enables direct molecular haplotyping of multiple previously known or unknown polymorphic markers in a single reaction.
To perform molecular haplotyping the chromosomes must be isolated prior to genotype analysis. Allele-Specific PCR (AS-PCR) is a popular method to selectively amplify a locus from the desired allele/chromosome from which genotypes data can be translated into haplotypes. However, available AS-PCR techniques are often leaky, leading to ambiguous results.
We have developed a novel AS-PCR technique to improve specificity. A non-extendable and exonuclease resistant competitor oligonucleotide is added to the reaction. The latter feature allows the use of proofreading polymerases permitting long-range AS-PCR (> 5kb). We called this novel strategy: Non-Extendable Exonuclease Resistant AS-PCR (NEER-AS-PCR). The amplicons can be used as template for genotyping assays. We have successfully used quantitative base extension reactions, using MassEXTENDTM, and demonstrated significant reduction in leakage when using 10-fold excess of the competitor. For multiple marker haplotypes, markers usually must be genotyped individually. To circumvent this issue we combined NEER-AS-PCR with base-specific cleavage of nucleic acid (MassCLEAVETM method). The NEER-AS-PCR product was subject to in vitro transcription and cleaved in four reactions. The cleavage products were analyzed by MALDI-TOF MS generating sequence specific mass signal patterns. Cross-comparison of theoretical and experimental mass patterns allowed haplotype determination (For more on MassCLEAVETM see poster #1590). We applied this method to the determination of molecular haplotypes in the CETP gene and provided a comparison to haplotypes imputed from individual genotyping. Because the analysis is not exclusive to known markers, novel variations associated with sub-haplotypes were discovered. Combined with MALDI-TOF MS this method allows the generation of comprehensive haplotype information at un-precedented speed and accuracy. We believe that this tool can potentially contribute to a better understanding of the complex etiology of cancer.

P107

ResonSense®: Simple linear probes for rapid fluorogenic PCR

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The rate limiting steps for high throughput & rapid Polymerase Chain Reaction (PCR) include sample preparation, thermal cycling, and assay signalling times. Improvements in sample preparation included new processing methods better suited for automation. Instruments with rapid temperature transitions times such as the Roche LightCycler® and Cepheid SmartCycler® can decrease the time for the PCR amplification from hours to tens of minutes using some fluorogenic chemistries. ResonSense® is a real-time chemistry based on the exchange of energy between DNA intercalators and fluor-labelled linear hybridisation probes. Their simple mode of action means that signalling times can be significantly reduced when compared to established real-time detection formats with added improvements and cost savings. BioGene's InSyte® is an open chemistry real-time PCR instrument capable of analysing ResonSense®, reducing strand-specific PCR to a matter of minutes. Here we describe the mode of action and benefits of this reporting chemistry.

ResonSense® is a trademark of the Defence Science and Technology Laboratory, an agency of the UK Ministry of Defence, International patents

P108

Improvement of gene expression analysis by RQ-PCR technology: addition of BSA.

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Real Time Quantitative PCR (RQ-PCR) using tumour specific RNA targets (e.g. fusion gene transcripts or FG) is used for diagnosis and detection of minimal residual disease (MRD) in bone marrow (BM) and peripheral blood (PB) of patients with acute lymphoblastic leukemia (ALL). However, several cDNA samples do not give appropriate amplification in RQ-PCR analysis. For example, in our laboratory 13.6 % of samples, of which 70% are of BM origin, are not amplifiable by RQ-PCR and therefore are defined as "poor quality" sample following Europe Against Cancer (EAC) criteria (*i.e.* copy number for *ABL* control gene amplification < 1000) established by an European network. In this context, it was decided to check if the failures of RQ-PCR in these samples were due to the presence of PCR-inhibitors. Dilution of cDNA, purification of cDNA on column and addition of an amplification facilitator, the Bovine Serum Albumin (BSA), in the PCR buffer were performed.
36 Peripheral blood and bone marrow samples were obtained from patients with ALL during follow-up. From EDTA PB and heparinized BM samples, mononuclear cells (MNCs) were collected by centrifugation on a Ficoll-Hypaque density step (Eurobio, les Ulis, France). RNA extraction was performed using TRIzol reagent according to the manufacturer's recommendations. After extraction

and isolation, RNA quality was assessed using the Agilent 2100 Bioanalyzer. For RT and PCR steps, EAC protocol was applied. RQ-PCR was performed on the 7700 machine (ABI, Foster City, CA, USA). Standard curves for quantification were performed using plasmid (Ipsogen, France) dilutions ranging from 10^5 to 10 copies for *TEL-AML1* or from 10^5 to 10^3 copies for *ABL* and allowed the determination of copy number (CN) for each transcript. To correct variations in RNA quantity, results of RQ-PCR amplifications were expressed as the ratio fusion transcript CN / control gene CN, also called normalised copy number (NCN).

The amplification of *ABL* and *TEL-AML1* genes were tested by RQ-PCR in duplicate after cDNA dilution in water and cDNA purification on column. The dilution involves a loss of sensitivity of 1 log and extensive purification of cDNA adds time and expense for sample preparation and leads to loss of target nucleic acid which prevents their use in MRD follow-up. Addition of 0.04% in PCR is the most effective way to avoid inhibition. BSA does not significantly affect PCR efficiency and does not affect the *ABL* CN detected in in amplifiable or "good quality" samples (*ABL* CN>1000). The majority of "poor quality" samples are sensitive to addition of BSA and can be considered as good quality samples according to EAC criteria allowing the clinical validation of the sample. Furthermore, addition of BSA has no effect on *TEL-AML1* copy number in samples which present correct amplification of the *ABL*. *TEL-AML1* expression is recovred in 70% of the cases with or without correct *ABL* amplification.

Our study shows that the addition of BSA to TaqMan-based RQ-PCR analysis improves *ABL* and *TEL-AML1* amplification of "poor quality" sample,. However, a large prospective study is warranted to definitively assess the use of addition of 0.04% BSA in CG and FG RQ-PCR in quantitative follow up of the MRD. Finally, the addition of BSA could be a good way of improving "poor quality" RNA (pharmaceutical and clinical samples) for gene expression analysis using Real Time Quantitative PCR.

P109

Trehalose is a potent PCR enhancer: reduction of DNA melting temperature and thermal stabilization of *Taq* Polymerase by the disaccharide trehalose.

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The PCR amplification of GC-rich templates is often difficult and can result in low yields or even absence of the desired amplicon. Several low-molecular compounds have been shown in the past to facilitate this phenomenon. The compatible solute trehalose thermostabilizes and thermoactivates several enzymes commonly used in molecular biology laboratories at 37°-42°C, i.e reverse transcriptase, restriction enzymes and low-temperature DNA polymerases. Here we introduce this compound for its application with high-temperature (PCR) polymerases.

In the absence of trehalose, the almost 90% GC-rich murine oxytocin receptor transcript is difficult to amplify by PCR. In the presence of trehalose, a 200-fold gain in amplification efficiency was achieved (Fig. 1). The enhancement is conferred by two different properties, which we identified by a real-time PCR approach. Firstly, trehalose decreases the melting temperature of DNA which is analog to the compatible solute betaine (Fig. 2), and secondly trehalose thermostabilizes the *Taq* polymerase against thermal denaturation (Fig. 3), as shown with Taq polymerase, that had been preincubated at 95°C for 90 min prior to PCR.

Conclusively, trehalose is a compatible solute which greatly facilitates the PCR of GC-rich templates by reducing the DNA melting temperature and thermostabilizing the *Taq* polymerase. This should prove useful in the PCR amplification of difficult templates.

Figure 1:

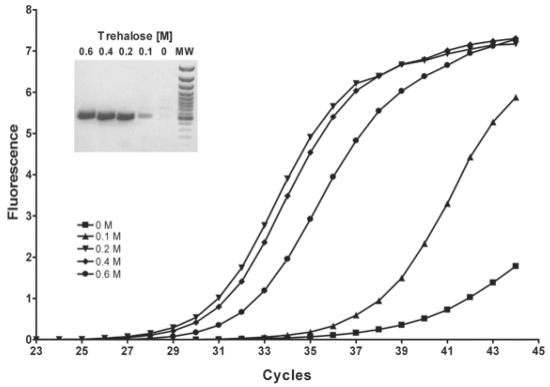


Figure 2:

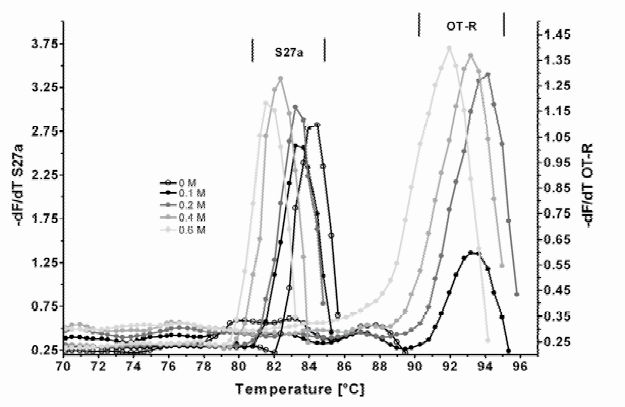
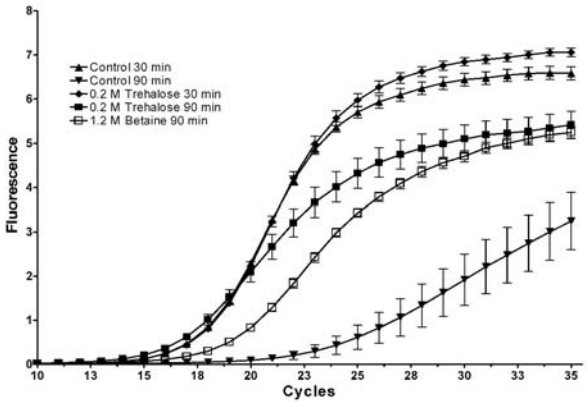


Figure 3:



P110

High confidence SNP scanning with the ds DNA dye, LCGreen I, in conjunction with high resolution melting analysis.

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Mutation scanning is an important tool for genetic research and clinical applications. Conventional methods include denaturing gradient gel electrophoresis (DGGE), single-strand conformation polymorphism (SSCP), and denaturing HPLC (dHPLC). The recently introduced dsDNA dye, LCGreen I, can be used to detect heteroduplexes by high-resolution amplicon melting analysis [Wittwer et al. Clinical Chemistry 49(6): 853-60 (2003)]. We have rigorously tested the sensitivity of this system for SNP heterozygote detection using a DNA mutation "Toolbox" [Highsmith et al. Electrophoresis, 20: 1186-94 (1999)]. The Toolbox consists of three different plasmids: M13, lambda, and pBR322, with regions averaging 40%, 50%, and 60% GC, respectively. For each plasmid, four possible nucleotides (A, C, G, or T) are interchanged at one position. Each construct alone is homozygous (a "wild type"); by mixing them 1:1, heterozygous genotypes can be obtained. For each plasmid, six primer pairs surrounding the SNP site were used for PCR and subsequent high resolution melting analysis. The PCR products ranged from 100 to 600bp in size and the SNP position was kept in the middle of the product. The tests were evaluated by a blinded investigator. For products smaller than 400bp, all possible heterozygotes were distinguished from their wild type homozygotes. For products between 400bp and 600bp, accuracy was 100% for the 50 and 60% GC constructs, and 86% for the 40% GC construct. To understand, why some heterozygotes were missed, the hypothetical melting behavior of the constructs was calculated through a melting prediction program [Lerman & Silverstein; Methods Enzymol, 155: 482-501 (1987)]. Also, known homozygotes and heterozygotes were shown to the blinded investigator for training on the amplicons that were missed. After this training and with knowledge of the predicted melting behavior another blinded study was performed. The sensitivity of detecting heterozygotes in the 40% GC plasmid increased to 96.9%. Interestingly, heterozygotes of G or C wild types resulted in 100% precise calls, whereby 57% of the A wild type and 43% of the T wild type failed. In summary, a total of 576 heterozygotes were analyzed and 563 were correctly called, resulting in an accuracy of 97.7%. LCGreen I in conjunction with high resolution melting analysis can be used with high confidence for SNP scanning with amplicons for up to 600bp.

P111

Rapid real-time PCR using a SuperConvection™ instrument.

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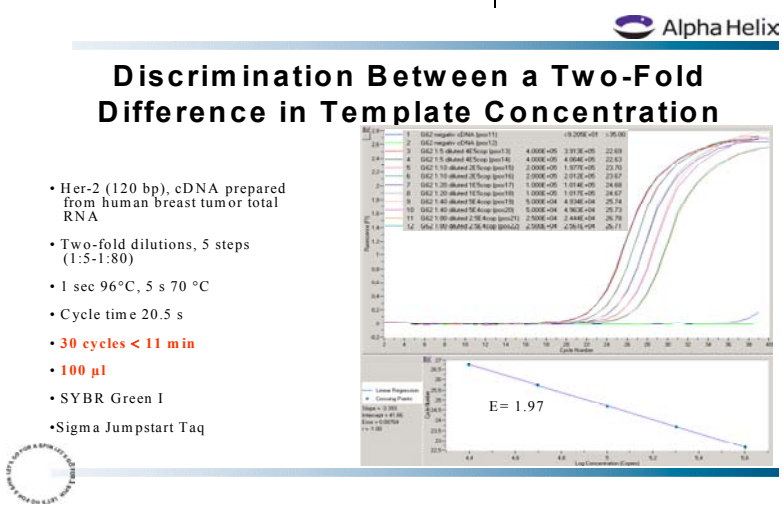
In certain clinical situations there is a need for rapid diagnosis of infections and other disorders. Typical cases are when patients are encouraged not to leave the clinic before results from detection of certain pathogens like HIV and Mycobacterium tuberculosis are obtained. Real-time PCR is a powerful method to detect pathogens. However, such an analysis either takes several hours to accomplish or requires miniature reaction vessels like narrow glass capillaries. Unfortunately, downscaling of reaction volumes results in a decreased scope for sensitivity since less template amounts can be used. Furthermore, working with capillaries requires special handling due to their fragility and shape. We have developed a multicolour detection real-time PCR-instrument that combines the advantages of full-scale (up to 150 µL) reactions and conventional plastic 0.2 mL PCR-tubes with retained quality benefits from rapid ramping.

The instrument typically obtains real-time PCR-results from 48 samples (50 µL) in 15-25 minutes including liquid handling and melting-curve analysis. This is achieved by performing thermal cycling at 7 000 x g, that is, under centrifugation. The parts of the sample having different temperatures will also differ in density. The high g-force and rotation effects induce a tremendous mass transfer in the sample, a process called "superconvection". In a superconvection instrument thermal homogeneity in the reaction vessel is achieved almost instantaneously which dramatically lowers the time for temperature equilibration. Data showing heating and cooling ramps of 5.0° and 5.8 ° C/s respectively in 50 µL are given. Results from a 100 µL Taqman assay with 30 seconds cycle time and a 40 cycles, 150 µL SybrGreen I in 29 minutes are presented. Also data on a 5 step dilution series of a Her2 template are shown to illustrate the discrimination of a two-fold dilution in template concentration. Intra assay variation with respect to Ct-values are also presented. In conclusion, increased mass transport induced by performing thermocycling under elevated g-force can improve real-time PCR with respect to cycle times in reaction volumes preferred in certain diagnostic situations. Ramping rates in such large volumes are at least as high as in capillary formats in which improved quality due to high ramping rates is well documented.



P112

Inhibition of *Taq* Polymerase and *MMLV* Reverse Transcriptase performance in presence of polyphenolic compounds: (+)-Catechine & Epigallocatechin Gallate (EGCG)

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Polyphenolic compounds present in many foods are known to have a preventive but also curative effect on carcinogenic progression by multiple effects on the cell physiology. A direct effect of polyphenols on the enzyme activity should therefore be considered. Herein we studied *in vitro* the inhibitory effects of two polyphenolic compounds (+)-Catechine & EGCG on the performance of the polymerase and reverse transcriptase, as a model for eukaryotic and viral enzyme activity. Since in real-time RT-PCR the reaction kinetics trajectory can be recorded, we compared several amplification histories obtained with or without polyphenols. Two different approaches of RT-PCR were adopted: A one-step RT-PCR approach (RT and PCR together in one run), where y_0 is showing the efficiency of the prior RT reaction. A two-step RT-PCR approach, where the mRNA was separately reverse transcribed, and polyphenols were added only into PCR. In each approach, reaction setups without any additional agent as a background control (n=8), and three serial dilutions of both polyphenols were performed: $1*10^{-5}$, $1*10^{-6}$, $1*10^{-7}$ and $1*10^{-8}$ M (n=3). We determined various parameters describing the enzyme properties derived from the sigmoidal shaped reaction trajectory, using an established four parametric sigmoid model (Tichopad et al., *Biotech. Lett.* 2002). Raw fluorescence data were fitted, where *f* is the function computed fluorescence at cycle *x*, y_0 is the background fluorescence, *a* the plateau height ($a = y_{max} - y_0$), *e* is the natural logarithm base, *x* is the cycle number, x_0 is the first derivative maximum (FDM), the second derivative maximum (SDM), and *b* describes the slope at x_0, representing the polymerase efficiency. Further the area under the melting curve peak (AUC) of a final PCR product was determined, representing the amount of amplified product. All statistics were done in SAS 8.02 using GLM, checking for differences between the groups. In one-step RT-PCR, only the effect of EGCG addition was significantly present as a decrease of final cDNA product after RT reaction. This is in accordance with known antiviral properties of EGCG. Decrease in PCR product was a consequence of decreased prior template cDNA. Employing two-step RT-PCR approach one can see an effect of both compounds on PCR performance. Parameters were altered in a sense of PCR inhibition and lower PCR efficiency. The range of added polyphenols was biologically relevant (10 nM to 10 µM) and able to inhibit the enzyme activities. In conclusion, our results suggest that polyphenols are suppressing the polymerase as well as reverse transcriptase activity *in vitro*. This may lead to the hypothesis, that organs exposed to polyphenols exhibit lower DNA replication and proliferation rate, as well as lower viral activity caused by retroviruses.

$$f = y_0 + \frac{a}{1 + e^{-(\frac{x - x_0}{b})}}$$

	One-step RT-PCR					
	a	b	x0 = FDM	y0	SDM	AUC
Catechine (p value)	0.8052	0.1858	0.9416	0.4086	0.5315	0.9273
EGCG (p value)	0.1051	0.5921	0.6113	*0.0294*	0.5171	*0.0462*
	Two-step RT-PCR					
Catechine (p value)	0.0815	*0.0015*	*<0.0001*	--	0.8843	0.4303
EGCG (p value)	0.0919	0.777	*<0.0001*	-	*<0.0001*	0.409

P113

Fast identification of probes and primers for intron spanning qPCR with the ProbeFinder web tool.

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The design of an efficient and reliable qPCR assay for a human gene is a complex task. We present ProbeFinder (see www.probeLibrary.com) a new web tool for for fast and easy selection of ProbeLibrary(TM) probes and the design of primers for qPCR of human genes. The ProbeFinder server finds the optimal design in three steps. First the position of introns is determined by a lookup in ENSEMBL or by the Exiqon intron predictor (which relies on a blast search against the human genome). Secondly, ProbeLibrary™ probes are matched to the sequence and primers are designed with Primer3. Finally the probes are ranked according to carefully selected rules ensuring the best possible qPCR. The rules favour intron spanning amplicons to remove false signals from DNA contamination, small amplicon size for reproducible and comparable assays and a GC content optimized for PCR. The optimal qPCR design is presented, and the user can select alternative solutions for genes with special requirements. Using the ProbeFinder web tool, a high quality qPCR assay based on Exqion's ProbeLibrary™ can be designed for virtually all human transcripts in a few minutes without the need for expert understanding of qPCR requirements.

P114

Four-color multiplex PCR assay for the simultaneous detection of four allelic variants in a closed tube using a single thermal cycler program

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Homogeneous allele-specific assays for single nucleotide polymorphism (SNP) detection offer several advantages over the more traditional techniques based on the use of the polymerase chain reaction (PCR). For instance, labor is significantly reduced and the chances of generating carryover contamination are diminished because no post-PCR handling is required. The 5' nuclease assay is one of the most widely used allele-specific homogeneous assays.

The assay takes advantage of the 5'→ 3' nuclease activity of Taq DNA polymerase to cleave fluorescently labeled allele-specific oligonucleotide (ASO) probes when they hybridize to PCR products during the PCR annealing phase. We developed a real-time multiplex assay for the simultaneous detection in of up to four allelic variants in one closed tube using a single thermocycling protocol and four probes each labeled with a different fluorophore. The assay combines the power of multiplex PCR with the specificity provided by ASO hybridization using the 5' nuclease assay format. We applied the four-color assay for the simultaneous detection of the factor V Leiden (FVL) G1691A and prothrombin (PT) G20210A mutations, the two most common known genetic risk factors for venous thrombosis in Caucasians. Human genomic DNA is prepared from whole blood using standard procedures. A 97 bp DNA sequence of the coagulation factor V gene is co-amplified with a 111 base pair DNA sequence of the coagulation factor II (prothrombin) gene using four PCR primers. In addition, PCR reactions included four differentially labeled ASO probes for the specific detection of the different FVL/PT G20210A genotypes. To evaluate the assay's performance characteristics, we performed a method-comparison study. Results generated with the four-color multiplex assay were compared with those obtained with a reference method. We analyzed 52 DNA samples with known FVL / PT G20210A genotypes that were previously genotyped with the reference method. We found a 100% concordance between the results generated by both methodologies. We conclude that the four-color multiplex assay is specific and reproducible for the detection of the FVL/PT G20210A mutations, and it can be easily adapted for the detection of other SNPs. The four-color assay, which will be useful for both molecular diagnostic and research laboratories, offers numerous advantages over more traditional methods for the detection of the FVL and PT G20210A mutations. The advantages include speed and simplicity of the method, reduced labor, reduced risk of cross-contamination, and higher throughput.

P115
PCR bias in multiplex real-time quantitative PCR

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We recently conducted a study using a novel multiplex real-time quantitative PCR assay for the diagnosis of trisomies of the chromosomes 18 and 21, where the difference in copy number is only 50%. To calculate the ratio of the two chromosomes under investigation, we implemented the efficiencies based ΔΔCT–method. The amplification efficiencies of the two sequences amplified were determined by dilution curve and the fluorescence increase method and shown to be equal and close to optimal. However, the ratios between the two chromosomes, as calculated by the formula were shifted towards 1.00. The multiplex amplification suffers from decreased ΔCT-values when the targets are present in similar numbers and we propose a modification for the ratio determination based on an empirically determined "relative" amplification efficiency.

We reanalysed the whole data-set acquired in the aneuploidy study, applying an efficiency of 2.2, instead of the measured efficiencies between 1.8 and 2.0. This procedure eliminates the discrepancy between calculated and actual ratio: The ratios calculated in this manner are close to 0.67 for trisomy 18 samples, and 1.50 for trisomy 21 samples, as is representative of the real situation.
A bias against the more abundant locus towards the PCR product ratio of 1:1 was already observed in our previous pilot study and had been reported for the later cycles of PCR. Also another multiplex PCR based method for the detection of trisomies recently described a decreased, non-linear relationship between chromosome dosage and the mean peak ratios of PCR products, as observed after capillary electrophoresis, with unclear cause.
Suggested reason is that the rate of formation of the more abundant PCR product declines faster than that one of the less abundant due to self-hybridisation of PCR products, thus preventing primer annealing to a fraction of the template. The higher self-inhibition during all PCR cycles for the more abundant product results in a successive decrease of the original difference.

P134
Haplotype Analysis using a Novel Real-Time Amplification Strategy on the MJ Research Opticon Continuous Fluorescence Detection System.

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Single nucleotide polymorphisms (SNPs) are well-recognized as markers for inter-individual differences in disease risk and treatment response in humans. For genes containing multiple SNPs, however, the haplotype (combination of SNPs on one chromosome) is often the principal determinant of phenotypic consequences. The human apolipoprotein E (*APOE*) gene encodes a protein that plays a key role in the transport and metabolism of plasma cholesterol and triglycerides. An individual's *APOE* haplotype influences the risk for cardiovascular disease and late-onset Alzheimer disease.
We have developed a protocol to rapidly genotype human *APOE* haplotype alleles using a novel real-time PCR strategy with SYBR Green I. The three common isoforms of apoE are encoded by haplotypes involving two diallelic SNPs of thymine/cytosine at nucleotide positions 3937 and 4075. We determined the *APOE* haplotypes based on the differential amplification of alleles using primer sets that contain specific terminal bases for SNP interrogation. DNA samples from 264 individuals were analyzed using the MJ Research DNA Engine Opticon Continuous Fluorescence Detection System. The frequencies of each apoE allele were: apoE2 - 6.6%, apoE3 - 79%, and apoE4 -14.4%. Our results are consistent with previous reports. Validation of the real-time PCR results by DNA sequencing analysis establishes the DNA Engine Opticon system as an efficient and reliable platform for genotyping.

Poster Session: siRNA

P116
Investigation of the adrenomedullin mechanism through small interfering RNAs in human cells

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Adrenomedullin (AM) is a regulatory peptide, which inhibits Ca^{2+}–dependent agonist-stimulated aldosterone secretion and stimulates the growth of adrenocortical cells cultured in vitro. AM acts via different receptor subtypes: L1 and calcitonin receptor-like receptor (CRLR). CRLR acts as selective AM receptor only in the presence of subtypes 2 and 3 of receptor activity modifying proteins (RAMPS), which generates two AM receptors, named AM_1 and AM_2. At present, two AM-receptor antagonists are available, $CGRP_{8-37}$ e AM_{22-52}, which, however, are non-selective for the various AM receptor subtypes, because they suppress all the biological effect of AM and completely displace the tissue binding of [^{125}I]AM. Hence, due to the non-selectivity AM receptor-antagonist, none is known on the role played by different receptor subtypes in the mediation of the various biological effect of AM.
Small interfering RNAs (siRNAs) induced gene silencing in mammalian cells has shown great promise as a tool to suppress the expression of specific genes with high specificity. RNA interference (RNAi) is a phenomenon in which a double-stranded RNA (dsRNA) reduces the expression of the homologous gene. In vivo dsRNA molecules are reduced by the action of an endogenous ribonuclease to siRNAs and are able to specifically silencing gene expression in mammals without induction of the unspecific interferon response pathway. We have selected siRNA for human GAPD, AM, L1, RAMP2 and RAMP3. Target sequences were aligned to the human genome database in a BLAST search to eliminate those with significant homology to other genes. In the present work we investigated the effects of the suppression of AM, L1, AM_1 and AM_2 expression obtained by transfecting cells with specifically designed siRNAs on the responses of human cells cultured in vitro (fibroblasts, prostate and NCI-H295).
The siRNAs were transfected into human cells and silencing were analysed for GAPD, AM, L1, RAMP2 or RAMP3 mRNAs. Total RNA were extracted and the expression level were determined for each experimental sample by real-time RT-PCR on the I-cycler with IQ SYBR GREEN Supermix. Our preliminary results have been show that the gene expression was decresed in all the cells studied. We are only beginning to apreciate the mechanistic complexity of this process and its biological implication. Further investigations, including the use of more human cell line, are under way to identify the specific physiological roles of AM and their receptors.

P117
Inhibition of gene *src* expression in *Xenopus laevis* quantified by real-time PCR

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c-Src is a member of the family of tyrosine-kinases, which are ubiquitous in every higher animal cells. The effects of c-Src on early development of amphibians are not clear up to now. Overexpression or underexpression has been a succesful way to study a function of a gene. We found that overexpression of the c-Src correlated in *X. laevis* with a defective embryo development (Takáč et al., Mol. Reprod. Dev. 50(1998) 410-419, Jonák, Mol. Reprod. Dev. 56(2000) 298-300) and a profound loss of cadherin and α-,β-,γ-catenins in tissues of aberrant organisms (Dvořáková et al., Folia Biol. (Prague) 46(2000) 3-10). For the inhibition of the c-*src*1 gene expression we used DNA knock-out constructs and RNA interference technology (RNAi) using specific siRNA (small interfering RNA). Three different siRNAs directed against the *src* gene were applied. They were targeted at kinase domain SH1, N-terminal region and SH3 domain. We injected siRNA or DNA constructs into fertilized eggs or two-cell embryos. RNA from two-, four- and six-day-old embryos was isolated. For the quantification of siRNA inhibition efficiency, the *src*-RNA level was determinated by quantitative real-time PCR (qPCR) with SYBR-Green detection. The results were compared with the RNA level of the following reference genes: elongation factor 1 alpha, GAPDH and N-tubulin. We also examined RNA levels of Yes, Lyn and Fyn kinases, because the *src* gene function might be substituted by other tyrosine kinases of the Src family. The morphological changes were documented by photography in parallel with qPCR results.

P118
Using Real-Time RT-PCR to Measure Gene Silencing by RNAi

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We have applied the recently discovered techniques for RNAi in mammalian cells (Elbashir et al, Nature 411: 494) to generate knockdown reagents for a set of 50 genes. In order to identify effective siRNAs (>80% knockdown), we used Applied Biosystems Assays-on-Demand Gene-Expression products to quantify mRNA levels. We evaluated these predesigned TaqMan reagents using a dilution series of cell line RNA and found that their sensitivity and reproducibility allowed reliable measurement of low transcript levels following knockdown.
Using Assays-On-Demand to measure knockdown, we tested candidates siRNA sequences in batches of three per gene until a successful siRNA was found. For a subset of genes, a 2nd potent siRNA was generated to allow confirmation of phenotypic effects using two independent knockdown reagents.

P119
Accelerating Drug Target Validation using LNA.

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Antisense has traditionally had a bad reputation for low success rates, toxicity (especially using phosphorthioate-modified oligonucleotides) and high dosage requirements. Locked Nucleic Acids (or LNAs) are a family of nucleotide analogues, which show higher hybridisation affinity and specificity characteristics compared to that of natural DNA combined with an increased nuclease resistance *in vivo*. Recent results have shown that chimeric LNA/DNA gapmers recruit RNase H effectively improving the efficacy of traditional antisense technology. In fact LNA antisense oligos are efficient in the low nanomolar range, comparable to siRNA's, making them highly attractive antisense tools for drug target validation. Moreover, LNA enables the use of short detection probes in qPCR, due to the significantly enhanced duplex thermal stability compared to DNA probes. In turn, this forms the basis for the design and production of a Human Probe Library comprising 90 dual-labeled LNA-containing probes, covering more than 99 % of the human protein-coding transcriptome. The unique combination of (i) LNA gapmer antisense oligos in gene knock-down, and (ii) verification of the knock-down phenotype at the RNA level by qPCR using short dual-labelled LNA probes, has allowed us to develop an improved, cost-effective functional genomics platform highly useful for drug target validation. The presentation will describe the LNA chemistry, antisense design as well as the unique features of the platform as demonstrated by human gene knock-down studies.

P120
Validation of siRNA knockdowns by real-time quantitative PCR

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Owing to its simplicity, wide dynamic range of quantification, sensitivity, and precision, real-time quantitative PCR (Q-PCR) is now widely used for the accurate evaluation of RNA expression levels. We describe here a real-time Q-PCR process to validate specific gene silencing mediated by short interfering RNAs (siRNAs), an important tool for studying protein function. Utilizing the naturally occurring post-transcriptional gene silencing mechanism of RNA interference (RNAi), synthetic siRNAs can be used to induce sequence-specific degradation of transcripts homologous to siRNAs. We have used synthetic siRNAs (Qiagen Inc., Germantown, MD) to study the function of 139 genes by transfection into mammalian cells in a high throughput manner. For siRNAs that elicit a specific phenotype, we have used TaqMan real-time Q-PCR assays to quantify the appropriate target transcript levels following RNAi. The TaqMan fluorogenic 5' nuclease assay has been found to be convenient, self-contained and sensitive. The TaqMan assay relies on a gene specific fluor tagged probe and the 5' nuclease activity of Taq DNA polymerase. As PCR product synthesis occurs, bound probe is digested liberating the fluor. Detection of this fluor is used to accurately quantify relative or absolute amounts of target. Semi-quantitatively both sensitivity and specificity are achieved in a practical manner unlike other assays (i.e. SYBR Green). After internal control normalization, our results show consistency both among TaqMan assay batches and sample replicates. In conclusion, this study presents a reproducible method to validate RNAi gene knockdow.

Poster Session: High Throughput & Array Technology & Cluster Analysis

P121
cDNA Microarray validation of small changes in gene expression by qPCR

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Quantitative gene expression studies are essential to understand normal and pathological processes. Using cDNA microarrays, a large number of genes can be analyzed at once, but usually the data can only be taken as a prediction of expression change and requires verification by other technology. In particular, qPCR has come into common use for this purpose as a more reliable technology. However, when changes in expression are less than 2 fold, qPCR can present some challenges. We present results of some physiological responses where transcriptional regulation may be of great functional significance, but of modest size. To reliably detect these changes, we are combining microarray analysis with an improved qPCR method. In order to screen a large number of genes using qPCR, we used a SYBRgreen I based assay. Because we are interested in detecting small changes (about 20% to 50%), the qPCR machine had to be carefully calibrated to ensure the same PCR efficiency across the plate. We found that conditions that yielded Ct values over 22 for a particular gene were less accurate and required calibration of the sample by using the appropriate dilution. We also used power analysis to calculate the number of replicates needed to detect changes of 10% with p-values less than 0.01. We used the $\Delta\Delta$-Ct method and calculate the t-test p-value to determine if the gene is differentially expressed (confidence interval at 99%).

(Supported by the National Institutes of Health, USA)

P122
The Applied Biosystems 7900HT Micro Fluidic Card System with Assays-on-Demand™ Gene Expression products.

John P. Bodeau, Sangita Parikh, Chris Grimley, Ian Harding, Adrian Fawcett, Kathy Lazaruk, Kathleen Shelton, Matthew Chan, Mark Wechser

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Joel R. Dufresne, Louis C. Haddad, Theresa Gerten
3M Bioanalytical Technologies Project, 3M Company, St. Paul, Minnesota, USA, 55144

The sequencing of the human genome has enabled gene expression researchers to design far more comprehensive studies than was previously possible. High-density microarrays now analyze the gene expression pattern of thousands of genes and identify genes of interest.
In combination with the Applied Biosystems Assays-on-demand™ products for human and mouse the Applied Biosystems 7900HT Micro Fluidic Card is an excellent tool for validating the "hits" generated from these gene expression studies, and thus, acts as a low-density, gene expression custom array. The Micro Fluidic card is designed for custom assay configuration using Assays-on-Demand™ Gene Expression assays and the ABI PRISM® 7900HT Sequence Detection System.
The Micro Fluidic card is fast and easy to use. Provides access to high-throughput, 384-well format without liquid-handling robotics. Innovative Micro Fluidic card technology facilitates the analysis of tens to hundreds of targets. This, combined with a well volume of only 2 µL (including both well- and channel volume), results in a more efficient use of biological sample – an important factor when only limited sample is available. The technology also uses reagents more efficiently, which reduces consumption and saves money.

P124
Gene expression profiling using clustering and functional annotations

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Microarrays can be used to identify candidate genes that might be involved in a disease or treatment of interest. However when validated by methods such as qPCR or *in situ* –hybridisation, microarray experiments are shown to produce substantial amount false positives. To facilitate the process, we suggest the use of bioinformatic validation methods prior to the wet lab validation. We measured gene expressions in rat *prefrontal cortex* at different time points during chronic antidepressant drug (Imipramine) treatment. Expression profiles of 1090 genes were clustered using the Self-Organizing Map (SOM). We then analysed whether genes belonging to the same functional group (which were formed using keywords from Swiss-Prot) were overrepresented in a defined cluster area. We found that several keywords showed correlation to a specific sub-area of the SOM. For example, genes annotated by the *serine protease* keyword had similar expression patterns. For the following validation steps we have then selected not only those genes that are the most differentially expressed but also those gene groups which showed co-regulation but were not necessarily evidently differentially expressed.

P125
Validation of microarray data from tumor sample biopsies by short LNA enhanced qPCR probes

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The newly developed array technology has made it possible to simultaneously monitor thousands of genes during tumor evolution and progression. To generate meaningful information from such large datasets, new methods have been developed to identify subclasses of genes having a coordinated expression change that may be used to classify biological samples. The expression level of the selected subset of genes may thus form a basis for molecular class predictors that are potentially useful for tumor staging and prediction of clinical outcome. Exiqon has developed a novel Human Probe Library, with a unique versatility, derived from the duplex stabilizing DNA analogue LNA (Locked Nucleic Acid), which enable detection of short (8 – 9 nt) frequently occurring "genetags" in human transcripts, so that each probe can be used to detect numerous different mRNA transcripts.
We have completed a direct comparison of expression data obtained with high density Affymetrix GeneChip arrays (Human Genome U133A) and qPCR using short LNA enhanced probes from the Human Probe Library by Exiqon™.
Total RNA from normal and tumour sample biopsies were used for synthesis of biotin labelled cRNA, which was hybridized to the GeneChip arrays after fragmentation. The arrays were stained with a streptavidin-phycoerythrin complex before scanning and analysis to quantify expressions levels of important diagnostic genes. The same total RNA samples were used to generate cDNA for qPCR quantification with the short LNA probes. The concordance between the outcomes of the different experimental approaches was found to be high. The respective advantages and disadvantages of the methods will be discussed.

P126
The real-time PCR microchip.

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We have developed the first prototype of a microchip for quantitative real-time PCR – the q-chip™. The prototype has 4 x 8 = 32 wells. In one design the wells are microfabricated in a transparent 500 µm thick SU8-polymer coated glass plate that is positioned on a heater chip. Each well is heated by a platinum heater (600 x 1600 micron), with heater line widths of 22 µm spaced to provide 80% light transmission for spectroscopic measurements. The

heaters and, hence, the wells' temperatures, are controlled individually. In an alternative design the wells are built in a 100 μm SU8-polymer layer on a silicon wafer. Each well has an integrated heater, temperature sensor, and a photodiode. The wells are thermally isolated with very low temperature cross-talk. Cooling is achieved by the samples spontaneously equilibrating to the temperature of surrounding air, which is typically of room temperature. The cooling process is rapid due to the large sample surface area. Sample temperature is controlled in the range 15 – 105 °C. With typical sample volumes of 100-200 nl the mean heat and cool ramp rates exceed 25 °C/sec. Samples and reagents are deposited with a 384 nanodispenser. Several approaches to prevent sample evaporation have been designed and they are currently being evaluated. Samples are illuminated from above using either laser or light emitting diodes. Fluorescence is measured either from below, using a PMT or the integrated photo diode array, or from above using a CCD camera. Microfilters are used to separate the luminescence from the incident light. The q-chip is scalable and the number of wells can be increased to analyze the expression of all the genes in a typical genome in a single run. For more information see www.wafergen.com.

P127

High Throughput DNA Methylation Analysis by Base Specific Cleavage of Single Strand Nucleic Acid and Analysis by Mass Spectrometry (MALDI-TOF)

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Gene silencing by epigenetic modifications like DNA methylation has become a field of growing interest in cancer research. So far large-scale studies were limited by the labor intensive and time-consuming phase involved in analysis of DNA Methylation.
Here we describe a novel approach suitable for high throughput DNA methylation analysis. This method requires bisulphite treatment of genomic DNA. Afterwards a non-methylation specific PCR product is generated followed by creating a single-stranded nucleic acid. This is cleaved in up to four separate reactions at positions corresponding to each of the four bases to generate a base specific cleavage pattern. The resulting fragments can then be analyzed by mass spectrometry.
These cleavage patterns allow discriminating methylated from non-methylated template. As a model system the IGF2/H19 region was chosen. In this model system we show the analysis of hemi-methylated DNA as well as completely methylated and non-methylated DNA.
The combination of mass spectrometry and base specific cleavage enables different applications in methylation analysis: Screen for methylation in amplicons of interest requires one reaction and allows analysis of 384 amplicons in 30 min. Analysis of exact methylation patterns in amplicons of interest requires 2 to 4 reactions (depending on the sequence) and allows analysis of 96 to 192 amplicons in 30 min. Analysis of methylation ratios; Detection limit ≈ 1%; Linear range ≈10 – 90%
The advantages of Mass spectrometry in terms of speed and accuracy over fluorescence based methods result in a throughput off around 384.000 CpG Sites per day for screening applications and 96.000 to 192.000 CpG sites per day for methylation pattern analysis.

P128

Gene expression in SIRS and septic patients monitored by real-time PCR using microfluidic cards

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Lipopolysaccharide (LPS) is the main inducer of shock and death in Gram-negative sepsis. Monocytes respond to LPS by inducing the expression of cytokines. In this context monocytes appear to have a central role in mediating the effects of LPS by interacting through a receptor called CD14. However, CD14 is a GPI-anchored cell surface protein. Until recently, little was known how LPS signals are transduced across the plasma membrane. Recent data indicate that members of the transmembrane toll-like receptor (TLR) family, called TLR2 and TLR4, are involved in LPS signalling. Since these receptors are supposed to be necessary for LPS-signalling and the finding that endotoxin tolerance in mouse peritoneal macrophages correlates with down-regulation of surface TLR4 expression prompted us to investigate the expression of proinflammatory cytokines and TLRs during sepsis. Blood samples were taken from healthy volunteers and from SIRS and septic patients from various stages of the disease using PAXgene collection tubes. RNA was prepared and the expression of 24 different genes was analysed by microfluidic cards on an ABI Prism 7900 HT. Our data indicate elevated expression of some TLRs in the blood of septic patients compared to healthy controls. Furthermore, elevated levels of anti-flammatory cytokines were observed, whereas expression of proinflammatory cytokines did not differ from the control group. The results of this study will be correlated with clinical parameters in order to find new predictive markers for the outcome of sepsis in human patients.
This work was supported by the BMBF and the DFG

P129

Novel Technology Permits Three Linear RNA Amplification Rounds and Yields Reproducible Microarray Data with Maintained Dynamics in Differential Expression Levels

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Gene expression experiments with microarrays are a powerful tool, yielding information about thousands of mRNA levels. Requirement of large RNA amounts (μg range) has limited these studies to readily available bulk samples. This contrasts with tiny samples from biopsies or more drastically with the low numbers of highly defined cells available with microdissection techniques (ng range). Studies with these samples are impossible without mRNA amplification.
Problems in this process are limited reproducibility and loss in dynamics of quantitative differences. Furthermore, analysis with high-density microarrays requires 10μg amplified mRNA. A minimal 500,000-fold amplification of

about 1ng RNA (≤100 cells) containing approximately 2% of mRNA, is just sufficient for one microarray hybridization. Commercial kits for linear amplification by *in vitro* transcription allow only two subsequent amplification rounds, barely reaching this threshold with about 1-millionfold amplification.
Our novel technology permits a third amplification round, resulting in more than 20-millionfold amplification, enabling multiple high-density microarray hybridizations. Data with high-density microarrays (Affymetrix HG-U133A) demonstrate high reproducibility after 2-rounds, after 3-rounds and even in cross-comparison of 2-rounds versus 3-rounds (100 versus 1ng RNA). Furthermore, 2-rounds amplified RNAs revealed consistency in the identification of differentially expressed genes, also evident in the quantitative conservation of very divergent levels.

P130
A Whole-Genome Linkage Disequilibrium SNP Map and Validated Assay Resource.

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We developed a set of 5' nuclease allelic discrimination assays to score single nucleotide polymorphisms (SNPs) with the aim of creating a reference map for use in candidate-gene, candidate region and whole-genome linkage disequilibrium (LD) mapping studies. The assays were validated by individually genotyping 90 DNA samples, 45 from African-American and 45 from Caucasian individuals, selected from the Coriell Human variation collection. Our goal is to define a set of >150,000 assays distributed across all the genes in the genome for SNPs of high heterozygosity in at least one population. Candidate SNPs were prioritized from the Celera RefSNP database which contains 4 million unique SNPs from combined Celera and Public SNP databases through a triage process that requires evidence of independent discovery of the minor allele. We selected SNPs on 27,007 Celera gene predictions, in a gene focused picket-fence with an average density of one SNP per 10 kb of gene length, including 10 kb upstream and downstream of the predicted gene boundaries. PCR primers and TaqMan® probes for the 5' nuclease assays were then designed by a software pipeline that picks oligonucleotide sequences and then screens the assays against the genome database for potential artifacts. Following genotyping 90 individuals, the performance of each assay is benchmarked against stringent criteria for background signal, adequate signal generation, and specificity. Our validation results showed that 94% of the SNPs tested in the population panels were polymorphic and about 90% of the assays passed our stringent performance criteria. Of those, 87% have minor allele frequencies ≥0.05 in Caucasian panel and 88% in African-American samples. These figures represent an extremely high SNP validation rate, and an unprecedented yield of common SNPs useful in LD mapping. Allele frequency data in the populations tested will be made available with the assays. The individual genotypes being generated have enabled us to identify blocks of LD and the haplotype diversity across all gene regions of the genome for these populations. This information is being used to refine the SNP set coverage.

P131
Cell based assays in 384 and 1536 well formats using MosQuito and the Acumen Explorer™

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Performance of real-time PCR (RT-PCR) requires accurate, low volume pipetting particularly in 384 well plate format. The MosQuito liquid handling system uses disposable tips which eliminate cross contamination when preparing RT-PCR reactions. In addition, its nanolitre dispensing capability enables reduction of total assay volume resulting lower reagent costs. There is a growing interest in combining whole cell assays with gene expression. In order to performs such experiments, there are a number of technologies that dispense cells into 384 well formats, but fewer that are able to dispense viable cells into a 1536 well plate.

In this study, we have used the MosQuito liquid handling system, which uses disposable pipettes, to dispense cells into 384 and 1536 well plates. We have then used the Acumen Explorer laser scanning fluorescence microplate cytometer to study proliferation of the cells over several days. We have shown that cell cytotoxicity and proliferation can be determined, as examples of whole cell assays. The results demonstrate that MosQuito can successfully dispense viable cells into 384 and 1536 microtitre plates suitable for detection and assay with the Acumen Explorer, which compliments its already established use in RT-PCR laboratories.

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Workshop Presentations

Friday 5 March 2004

12:00 – 14:00 Registration

14:00 – 14:30 Welcome & Opening of the Application Workshop by Prof. M. Kubista

Lectures by the Workshop participating Companies
Lecture hall HS 15

14:30 "The new LightCycler 2.0: an advanced multi-channel system for rapid real-time PCR."
Oliver Geulen, Roche Diagnostics, LightCycler Devolpment Group, Germany

14:50 "Complete Solutions for Real-Time PCR approaches – Bio-Rad Real-Time PCR Systems."
M. Neusser & Luis Ugozzoli, Bio-Rad Laboratories GmbH, 80939 München, Germany

15:10 "Haplotype Analysis using a Novel Real-Time Amplification Strategy on the MJ Research Opticon Continuous Fluorescence Detection System."
Chas Andre, MJ Research Inc., Waltham, MA, USA

15:30 – 16:00 **Coffee break**

16:00 "New tools for genetic research: Whole genome microarrays and customized low density solutions"
Thomas Rygus & Thomas Schild, Applied Biosystems, Germany

16:20 "Multiplexing your assay, from simplex to fourplex."
Fabrice Magnino, Stratagene Europe, Amsterdam, The Netherlands

16:40 "Optimizing Assays in real time amplification."
Thomas Kaiser, Corbett Research R&D, Australia sponsered by Pyrosequencing, Sweden

17:00 "Normalization using the F3 Channel of the Lightcycler- a New Reporter Enables Multiplexing with 5'Nuclease Probes."
"A Two-Color TaqMan Assay on the LightCycler 1.2."
Mary Katherine Johansson & Brian Erich Caplin, Biosearch Technologies, Novato, CA, USA

17:20 "The effect of consumable type on the sensitivity and reproducibility of QPCR."
Sarah Freshwater, ABgene, Blenheim Road, Epsom, UK

17:40 "Test Systems for Fast and Automated Molecular Diagnostics."
William A. McMillan, Cepheid

18:00 **Open evening & Visit the Nightlife of Freising** (www.freising.de)

"The new LightCycler 2.0: an advanced multi-channel system for rapid real-time PCR."

Oliver Geulen, Roche Diagnostics, LightCycler Devolpment Group, Germany
(oliver.geulen@roche.com)

The Roche LightCycler technology set the standard for rapid, sensitive and accurate real-time PCR. Now, with the updated and improved LightCycler 2.0 system extremely fast online quantification of PCR products is combined with product identification and (automated) genotyping due to the Melting-Curve principle.
Every approach to quantification relies on a comparison to known standards and is influenced by differences in efficiency. Polynomial vs. linear regression of a standard curve combined with the accuracy of statistically valid amounts of standard replicates yields highest reproducibility for absolute quantification. The analysis of gene expression studies is substantially

improved by new features of the relative quantification software and provides now the flexibility for different depth in result interpretation: different approaches to qPCR (e.g. setting efficiency equal to two or efficiency correction) allow different levels of accuracy according to the requirements of an experimental approach.
With six detection channels and increased reaction volumes the LightCycler 2.0 instrument provides all prerequisites and the flexibility required for complex PCR, including multiplex applications. Advanced data and user management capabilities combined with enhanced analysis modules enable the implementation of control functions for reliable and accurate data analysis.

"Complete Solutions for Real-Time PCR approaches – Bio-Rad Real-Time PCR Systems."

M. Neusser & Luis Ugozzoli Bio-Rad Laboratories GmbH, 80939 München, Germany (Marcus_Neusser@Bio-Rad.com)

Real-Time PCR Systems are powerful tools for gene quantification and SNPs detection. Several iCycler iQ / MyiQ features used for different real-time applications (melt curve, gradient, software for SNPs detection, and absolute and relative gene quantification) will be shown. Futhermore, we will demonstrate how the kinetics of a PCR amplification can be followed in real-time during a PCR cycle, and how you can use this information for reaction optimization. Complete solutions for reverse transcription and amplification of target genes will be demonstrated with Bio-Rad reagents and supermix tools.

"Haplotype Analysis using a Novel Real-Time Amplification Strategy on the MJ Research Opticon Continuous Fluorescence Detection System."

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Single nucleotide polymorphisms (SNPs) are well-recognized as markers for inter-individual differences in disease risk and treatment response in humans. For genes containing multiple SNPs, however, the haplotype (combination of SNPs on one chromosome) is often the principal determinant of phenotypic consequences. The human apolipoprotein E (*APOE*) gene encodes a protein that plays a key role in the transport and metabolism of plasma cholesterol and triglycerides. An individual's *APOE* haplotype influences the risk for cardiovascular disease and late-onset Alzheimer disease.
We have developed a protocol to rapidly genotype human *APOE* haplotype alleles using a novel real-time PCR strategy with SYBR Green I. The three common isoforms of apoE are encoded by haplotypes involving two diallelic SNPs of thymine/cytosine at nucleotide positions 3937 and 4075. We determined the *APOE* haplotypes based on the differential amplification of alleles using primer sets that contain specific terminal bases for SNP interrogation. DNA samples from 264 individuals were analyzed using the MJ Research DNA Engine Opticon Continuous Fluorescence Detection System. The frequencies of each apoE allele were: apoE2 - 6.6%, apoE3 - 79%, and apoE4 -14.4%. Our results are consistent with previous reports. Validation of the real-time PCR results by DNA sequencing analysis establishes the DNA Engine Opticon system as an efficient and reliable platform for genotyping.

"New tools for genetic research: Whole genome microarrays and customized low density solutions"

Dr. Thomas Rygus, Dr. Thomas Schild, Applied Biosystems (Germany)

The sequencing of the human genome has enabled gene expression researchers to design far more comprehensive studies than was previously possible. The new Applied Biosystems Expression Array System includes, on a single microarray, probes to detect an annotated and fully curated set of more than 30,000 human genes. The system, based on proprietary chemiluminescent technology, has been designed to detect a greater number of genes, including those expressed at lower levels, with higher sensitivity and specificity while using less biological sample. For more detailed studies low density microarrays may be used for validating the "hits" generated from these gene expression studies. The Applied Biosystems 7900HT Micro Fluidic Card provides an alternative option for rapid gene expression analysis. In combination with the Applied Biosystems Assays-on-Demand™ products for human and mouse it is an excellent new tool for validating the "hits" and quantify the gene expression level with the accuracy of real-time PCR, and thus, acts as a low-density, gene expression custom array. The 7900 HT Micro Fluidic Card is designed for custom assay configuration using Assays-on-Demand™ Gene Expression assays and the ABI PRISM® 7900HT Sequence Detection System.
The 7900 HT Micro Fluidic Card saves time and reduces labor-intensive steps while offering high flexibility. On one card 12 to 384 different genes can be analyzed for up to eight samples. This innovative Micro Fluidic Card technology facilitates the simultaneous analysis of tens to hundreds of target genes. In combination with a very low well volume (2 µL, including both well- and channel volume), this results in a more efficient use of biological sample – an important factor when the amount of sample is limited. The technology also uses reagents more efficiently, which reduces consumption and saves money.
In this talk, the new Applied Biosystems Expression Array System will be demonstrated, as well as the principles of the Micro Fluidic Card technology and first results.

"Multiplexing your assay, from simplex to fourplex."

Fabrice Magnino, Stratagene Europe, Amsterdam, The Netherlands

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QPCR has revolutionised projects requiring nucleic acid quantification, especially those involving limited and irreplaceable tissue samples. Using QPCR, it is now possible to do such things as routinely quantify gene expression levels with high precision, perform reliable single nucleotide polymorphism detection, and rapidly quantify the level of DNA methylation.

Although many scientists now turn to QPCR as a more sensitive and efficient method of nucleic acid quantification, many still believe that multiplex QPCR (analyzing multiple targets in the same sample) requires too much optimisation to be a practical approach. At Stratagene, we believe that any scientist can develop successful multiplex QPCR assays by matching the right tools with a thorough understanding of the basics of QPCR assay design and optimization.

Stratagene is a leading provider of innovative solutions for QPCR research. We offer a full range of products to assist in designing, analyzing, and validating multiplex QPCR assays. Our Mx4000® and Mx3000P™ real-time systems are designed to achieve unparalleled results using Brilliant® QPCR reagents (1-2 targets) or our new Brilliant® multiplex master mix (3-4 targets) with probes of practically any chemistry.

We will also introduce our new fast QPCR technology that utilizes a novel, non-*Taq* enzyme. The FullVelocity™ QPCR and QRT-PCR master mix kits greatly reduce overall QPCR run times with probe-based chemistry while providing high sensitivity, reliability and reproducibility. This new enzyme technology withstands rapid cycling conditions and can be used on both conventional 96-well block cyclers such as our Mx3000P instrument, as well as other platforms.

Optimizing Assays in real time amplification."

Thomas Kaiser, Corbett Research R&D, Australia
sponsered by Pyrosequencing, Sweden

"Normalization using the F3 Channel of the Lightcycler- a New Reporter Enables Multiplexing with 5'Nuclease Probes."

Mary Katherine Johansson[1] and Brian Caplin[2]

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Many real-time PCR assays, such as gene quantification, benefit from multiplexing. Multiplexing allows the simultaneous detection of multiple targets. Normalization is most efficiently done using an internal control, for example by using a housekeeping gene for RT-PCR expression profiling. Pulsar-650 is a new reporter dye that enables multiplexing with taqman probes on the Lightcycler® real-time PCR platform. Pulsar-650 is efficiently excited with the fixed 470 nm excitation source and has emission that is detected in the F3 channel. Thus, two different 5'nuclease probes can be analyzed together with Pulsar-650 in the F3 channel and FAM in the F1 channel.

"The effect of consumable type on the sensitivity and reproducibility of qPCR."

Sarah Freshwater[1], Anne van der Valk[1] , Meg O'Shaughnessy[1], Simon Ng[1], Simon Baker[1, 2]

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The optimisation of quantitative or real-time PCR reactions can be laborious and time-consuming. The focus of this preparative work is often on the concentration of the various reagent components and assessment of the quality of template. However, we have shown that the choice of PCR plate and seal also has a significant effect on the reproducibility of the results and thus indirectly on the efficiency and applicability of the assay under development.
Opaque white microwell PCR plates or tubes are commonly used to maximise signal for many fluorescent applications, but we have now shown that they have a positive and quantifiable effect during the detection phase of QPCR. The white plates have a particularly noticeable effect at low copy number, raising the signal-to-noise ratio compared to transparent plates. The improvement in endpoint values and Ct values outweighs the minor inconvenience of a non-transparent tube format. We postulate that these improvements are a consequence of an increase in fluoresence being reflected back to the detector rather than being dissapated through interaction with the PCR block itself. While the performance of the detector is also improved by the optical clarity of the seal or cap, the efficiency of microplate sealing will have a demonstrable effect on evaporation of the reagents. Sensitivity was found to be reduced by 0.5Ct values and endpoint values reduced by 200RFU for each µl of water removed from reactions in simulated evaporation experiments.
For minimising variation in QPCR applications, ABgene® recommend the use of cleanroom-manufactured consumables to minimise risk of contamination. The PCR plate should be made of opaque white polypropylene for highest sensitivity and consistency, coupled with optically clear heat seals or high-quality adhesive QPCR seals.

"Test Systems for Fast and Automated Molecular Diagnostics."

William A. McMillan, Cepheid

Molecular testing for the diagnosis of bacterial or viral infections from raw clinical specimens requires complex, multistep procedures to release and isolate nucleic acids before PCR amplification. Laboratory bench-top sample preparation procedures are very labor- and equipment-intensive, and are prone to operator or equipment errors that lead to erroneous, but believable results. The need for automation has led to

the development and introduction of robotics-based laboratory instruments with discrete operations that simulate the basic functions of a laboratory technician.

Cepheid's GeneXpert family of products combine microfluics cartridge-based sample preparation with the amplification and detection functions performed by our I-CORE® modules in an integrated, automated DNA analysis instrument. These products are designed to purify, concentrate, detect, and identify targeted DNA sequences, taking unprocessed sample to result in less than 30 minutes. The I-CORE four color fluorescence capability enables true total internally controlled reactions based on realtime PCR, so no external controls are required. On-board dried reagents are preassembled into the cartridge chambers, are reconstituted at the end of sample preparation, and provide good ambient temperature stability. A unique integrated ultrasonics system is capable of lysing bacterial spores and vegetative cells in 15 seconds. Different cartridge types have been designed each for a different family of organisms and/or sample type, including bacteria from aqueous based media (swabs, CSF, wet bioaerosols), RNA from various specimens (CSF, tissues, blood), and combinations such as needed to prepare both bacteria and viruses from the same sample.

The GeneXpert technology and utility of the total internal control scheme have been extensively tested and validated for the detection of *Bacillus anthracis* in mail sorting centers of the United State Postal Service. This assay detects 2 virulence-associated plasmids, an internal control, and a sample preparation control and has a limit of detection is several orders of magnitude below the LD50 for anthrax. Over 30,000 specimens have been tested to date with no false positives. Cepheid is currently developing GeneXpert products for Group B Strep for testing at labor and delivery, a stat enterovirus test in CSF, breast cancer sentinel lymph node testing in the operating room, and a semi-quantitative CML test for minimal residual disease testing. These products form the basis for an expanding menu of products in infectious disease and cancer diagnostics.

Workshop agenda and organization

The 80 participants will be divided into two groups of 40 persons in each. One group will have seminars covering practical aspects of qPCR and upstream processes, before lunch and hands-on experiments and data-analyses after lunch. The other group will have hands-on before lunch and seminars after.

Part 1: Introduction

Part 2: Quantification Strategies

Covering Absolute, Relative and Comparative Quantification. How standard curves are established and the effects of efficiency estimations for quantification results. Several examples are presented that demonstrate the effects of erroneous efficiency estimations.

Part 3: Nucleic acids extraction

The most common extraction methods of nucleic acids and their pros and cons.
Quality control of nucleic acids for qPCR.

Part 4: Reverse Transcription

The available methods and strategies for reverse transcription as well as practical considerations when performing RT.

Hands-on:

Each group of 40 persons is divided so that a **small group of approximately 4 persons is stationed at each instrument.** This group sets up, programs and starts an experiment on that machine. The groups will the rotate and get to see experimental data on the next station/instrument and see how software, setup and analysis works on that instrument. Also another type of experiment is performed on each instrument. The groups rotate until they have seen all experiment/instrument types.
One instructor will be available for each station as well as company representatives to help assist the programming of the software and answer any questions.

The instruments and experiments:

LightCycler - SNP detection using FRET probes
ICycler - Duplex reaction using Molecular Beacons
myCycler - SYBR Supermix- insitu calibration
Stratagene - BEBO and BOXTO, new dyes for qPCR
Opticon 2 - SYBR Green siRNA knockdown
SmartCycler - SYBR Green
ABI Prism 7900 - TaqMan Assay
Rotorgene 3000 - QZyme (BD Biosciences)

For setup of the experiments the participants will have approximately 1 hour and then approximately ½ hour on each platform.

Workshop participating companies

Abgene

http://www.abgene.com/

ABI Instruments
http://www.appliedbiosystems.com/products/productdetail.cfm?ID=42

Bio-Rad Instruments & Reagents
http://www.biorad.com/icycler/

Biosearch-Technologies Probes
http://www.biosearchtech.com/

Cepheid
http://www.cepheid.com/
http://www.smartcycler.com/

Eppendorf qPCR Reagents & Instruments
(centrifuges & pipetting sets)
http://www.eppendorf.com/mastercycler/de/

Corbett Research Instruments
Rotor-Gene and CAS-1200 Robot

http://www.corbettresearch.com/

Roche Instruments & Reagents

LightCycler System Family

LightCycler & LightCycler 2.0

Stratagene Instruments

http://www.stratagene.com/QPCR/

MJ Research Instruments

http://www.mjr.com/html/instruments/opticon/index.html
http://www.biozym.com/

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