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

1st International qPCR Symposium & Application Workshop

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)

Roche Applied Science, Penzberg

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."

Chas Andre, Ph.D.[1], Fan Chen, Ph.D.[1], Vicki Pandey[1], Rich Kurtz, Ph.D. [2], and David Batey, Ph.D. [2]
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[1]MJ Bioworks, South San Francisco, CA; [2]MJ Research, South San Francisco, CA

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

Fabrice.Magnino@Stratagene.Com

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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[2] School of Biological and Chemical Sciences, Birkbeck, University of London, UK

SarahF@abgene.com

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.

Seminars:

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 <u>preliminary</u> 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/

PYROSEQUENCING

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/