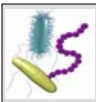


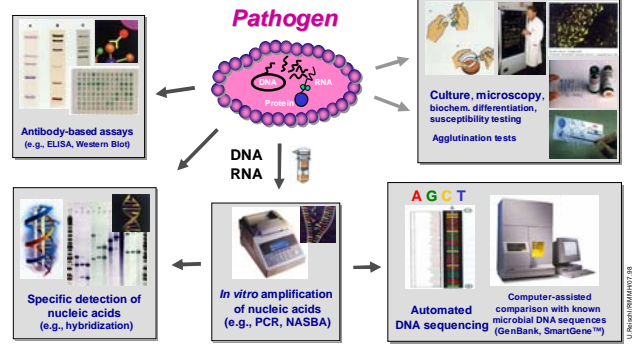

qPCR 2007 26 – 30th March 2007
Symposium & Exhibition & Workshop
 3rd International qPCR Event, Technische Universität München,
 Freising-Weihenstephan, Germany

Real-time PCR in Diagnostic Microbiology - a review on 9 years of R&D in an academic environment




Dr. Udo Reischl
 Institute of Medical Microbiology and Hygiene
 University Hospital of Regensburg
 Regensburg, Germany



**Principles of
Molecular Diagnostics**
qPCR 2007 26 – 30th March 2007
Symposium & Exhibition & Workshop
 3rd International qPCR Event, Technische Universität München,
 Freising-Weihenstephan, Germany



Antibody-based assays
 (e.g., ELISA, Western Blot)

Specific detection of nucleic acids
 (e.g., hybridization)

In vitro amplification of nucleic acids
 (e.g., PCR, NASBA)

Automated DNA sequencing
 Computer-assisted comparison with known microbial DNA sequences (GenBank, SmartGene™)

Culture, microscopy, biochem. differentiation, susceptibility testing, Agglutination tests

AGCT




U. Reischl/Mannheim 2007

Microbiological Practice



Hunting for the bugs ...

U. Reischl/Mannheim 2007



**Reasonable Indications
for requesting PCR-based tests**


- **When the highly desired same-day-results can not be obtained by conventional diagnostic procedures (i.e., microscopy, serology, antigen detection)**

and/or:

- testing specimens from antibiotic pre-treated patients
- for rapid detection of slowly growing, fastidious, or "viable but not culturable" pathogens
- when molecular detection of known resistance genes or pathogenicity factors contribute to the reliability of diagnostic reports in the case of unclear phenotypic results
- for rapid epidemiological strain typing (i.e., MLST)

Reischl, U., Indikatoren für die molekulare Diagnostik - Bakterien, Pilze, Eukaryonten. In: Leitfaden Molekulare Diagnostik (Thiemann, Cullen, Klein, Eds.), Wiley-VCH, Weinheim, 2006, pp.175-183.

U. Reischl/Mannheim 2007





Once upon a time...



LightCycler™
 PCR Perfected!

...in August 1998

U. Reischl/Mannheim 2007





August 1998

**Enjoy the benefits of
Boehringer Mannheim's
innovative high speed PCR
and analysis instrument for research**



Perform High Speed Thermal Cycling
 Highly efficient heat transfer allows up to 16 reactions for temperature sensitive and very fast 30 s at volume plus replicates in reaction vessels. Thermocycling cycles (including PCR product analysis) are completed in less than 30 minutes! This makes the LightCycler the fastest thermal cycler in the world.

Analyze melting characteristics
 Precise thermal transfer and rapid measurements allow you to analyze melting characteristics for each capillary sample. Based on melting temperature analysis you can verify your amplification and detect mutations on the computer screen. Flexible software programming even allows you to perform melting curves between cycles.

Quantify results quickly and accurately
 Rapid fluorescence readings - perform measurements every cycle for every sample in just 20 msec. This data acquisition process provides you on-line with accurate quantification graphs!

Elimination of Contamination Risk
 Amplification and fluorescence detection occur in the same closed tube. Contamination is eliminated because the PCR tube is never opened after amplification.

Monitor Fluorescence On-line and Real-time
 Monitor reactions on-line, while user-friendly software displays the amplification results in real time after each cycle. On-line detection throughout the entire process permits easy and accurate kinetic quantification.


LightCycler™

U. Reischl/Mannheim 2007

Enjoy the benefits of **Boehringer Mannheim's innovative high speed PCR and analysis instrument for research**

... some visionary statements

Haven't decided yet? Just think about it. In the time it has taken you to read about and consider the new **Boehringer Mannheim LightCycler**, one of your PCR reactions could be complete, maybe two. For more information, contact your local **Boehringer Mannheim** representative to see how the **LightCycler** can make a real difference in the way you do your research.

LightCycler™

U-Research/IMM/03.2007

Technological Evolution

Phenol / chloroform EtOH precipitation

Affinity columns

MagiK Pure LT

Sample preparation

In vitro Amplification

DNA-Sequencing

U-Research/IMM/03.2007

Real-time PCR in Diagnostic Microbiology

Added value by using Real-time PCR instead of traditional block cycle PCR:

- Speed
- Melting curve analysis
- Option for Nested-PCR
- Quantitative results
- Multiplexing (?)

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Evolution of the Real-Time PCR Platforms

or how technology meets the various demands of molecular diagnostic laboratories...

U-Research/IMM/03.2007

Current Spectrum of Real-Time PCR Platforms

The remarkably creative genius behind real-time PCR technology:

AACC Hall of Fame
Carl T. Wittwer
 Professor of Pathology at the University of Utah Medical School and Director of Flow Cytometry and the Advanced Technology Group at ARUP, Salt Lake City.

Real-time PCR

Rapid Cycle Real-time PCR

Hybridization Probes

Melting Curve Analysis

High Resolution Melting Analysis

U-Research/IMM/03.2007

Evaluation of Diagnostic Protocols in Clinical Bacteriology

Evolution of the LightCycler Real-Time PCR Systems

Options for high sensitivity or high throughput testing within a common assay format:

DNA and/or RNA

LC 1.0 (20 µl)

LC 1.x (20 µl)

LC 2.0 (20 µl, 100 µl)

LC 480 (5 µl, 20 µl, 100 µl) (96-well, 386-well)

LightCycler University

http://www.idahotech.com/lightcycler_us

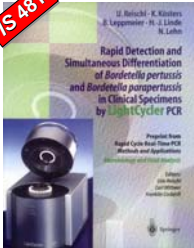
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Evaluation of Diagnostic Protocols in Clinical Bacteriology

- Bordetella pertussis / parapertussis -

Comparative study on diagnostic culture vs. PCR-based detection:

IS 481



Culture result	No. of patients (n = 208)			
	<i>B. pertussis</i> PCR		<i>B. parapertussis</i> PCR	
	Positive	Negative	Positive	Negative
Positive ^{a)}	20	0	10	0
Negative ^{b)}	2	164	8	190

^{a)} Includes 2 patients whose samples were both positive by PCR and culture for *B. pertussis* and *B. parapertussis*.
^{b)} Includes 2 patients whose samples were both positive by PCR and negative by culture for *B. pertussis* and *B. parapertussis*.

INSTAND-566 > WK_05/04-04 U. Reichel / RMMH/08.2004

Medizin-Schock

Es ist passiert +++ Erstes Bakterium völlig resistent gegen Antibiotika +++ Es heißt Staphylococcus aureus +++ Es hat in Hongkong bereits eine Frau getötet +++ Ein Mikro-Biologe sagt: Es hat unsere letzte Verteidigung durchbrochen

Resistente Bakterien Die größte Gefahr lauert in Kliniken

U. Reichel / RMMH/02.05

Evaluation of Diagnostic Protocols in Clinical Bacteriology

MRSA - Diagnostic Workup

Rapid

Microscopy (Gram stain)
 Blood agar + AB Discs
 Mannitol Salt agar
 Oxacillin Mannitol Bouillon

day 0

S. aureus
 Coagulase/MRSA Agglut.-test
 Catalase Prod.
 MH-Agar + AB Discs
 Oxacillin Screen plate

day 1

Antibiogram
 MRSA / MSSA
 Reading of AB Discs & Oxa screening plate
 Tubed coagulase

day 2

Decisive winning margin with respect to timely initiation of selective and efficient isolation measures by the availability of "same day results"

MRSA Nasalstrichbesteck - PCR
 PCR-Infektiediagnostik am selben Tag nur bei Probeabgabe vor 14:00 Uhr (Mo. bis Fr.)
 Ergebniszeitpunkt: heute, Taglier, Infektiochemie; heute, Taglier, Infektiochemie (L2/A.4411)

3 h

MRSA PCR pos. / neg.

Staphylococcus PCR
 mecA PCR
 8-Globin PCR

Evaluation of Diagnostic Protocols in Clinical Bacteriology

MRSA PCR-Workflow

same day results

TAT - 2 h
TTR - 2.5 h

Nasal swab → Prot K Incubation → Standardized DNA preparation (MagNA Pure LC) → DNA → LightCycler PCR

~ 3 h (110/70) for 1 to 6 samples

10:00 → 14:00 → 17:00


Staphylococcus
 mecA PCR
 SCCmec PCR
 lukS (PVL) PCR

RESULT

MRSA 111e
 SP: DNA isolation, AMP: Amplification, DP: Detection, INT: Interpretation of results

Roche SEPTI Fast®

Rapid and sensitive detection of bacterial and fungal sepsis from EDTA blood samples.



Evaluation of Diagnostic Protocols in Clinical Bacteriology

SeptiFast

Concept of the Roche SeptiFast® Test

Day 1	Day 2	Day 3	Day 4
Blood Culture	Gram	Species	Resistance
6 h PCR	Gram, Species, Resistance	adjustment	culture
2-3 days			

U. Reichel / RMMH/11.2006

SeptiFast Evaluation of Diagnostic Protocols in Clinical Bacteriology

Species Coverage of the Roche SeptiFast® Test

DNA from the following bacterial and fungal species can be detected and identified by the Roche LightCycler® SeptiFast® test within a timeframe of 6 h:

Gram-Negative	Gram-Positive	Fungus
<ul style="list-style-type: none"> • <i>Escherichia coli</i> • <i>Klebsiella (pneumoniae / oxytoca)</i> • <i>Serratia marcescens</i> • <i>Enterobacter (cloacae / aerogenes)</i> • <i>Proteus mirabilis</i> • <i>Pseudomonas aeruginosa</i> • <i>Acinetobacter baumannii</i> • <i>Stenotrophomonas maltophilia</i> 	<ul style="list-style-type: none"> • <i>Staphylococcus aureus</i> • CoNS (Koagulase-negative Staphylokokken) • <i>Streptococcus pneumoniae</i> • <i>Streptococcus spp.</i> • <i>Enterococcus faecium</i> • <i>Enterococcus faecalis</i> 	<ul style="list-style-type: none"> • <i>Candida albicans</i> • <i>Candida tropicalis</i> • <i>Candida parapsilosis</i> • <i>Candida krusei</i> • <i>Candida glabrata</i> • <i>Aspergillus fumigatus</i>


- Detection of the 25 most important pathogens found in blood stream infections
- Identification of 20 groups relevant for decision making
- Covering approx. 90 % of the nosocomial blood stream infections

U. Reusch/IRMM/03.2007

SeptiFast Evaluation of Diagnostic Protocols in Clinical Bacteriology

Concept of the Roche SeptiFast® Test

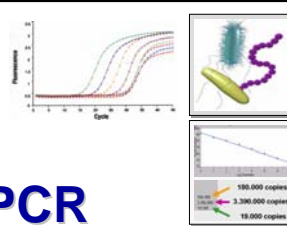
Multiplex and multi-channel concept: LightCycler® 2.0 instrument (CE-IVD):



Channel	Gram-positives	Gram-negatives
Channel 610	<i>S. spp.</i> from SML (S10)	<i>Proteus mirabilis</i> <i>Serratia marcescens</i> <i>Acinetobacter baumannii</i>
Channel 640	CoNS from SML <i>Staphylococcus aureus</i>	<i>Klebsiella pneumoniae</i> <i>Klebsiella pneumoniae</i> <i>Enterobacter aerogenes / cloacae</i>
Channel 670	<i>S. spp.</i> from SML (S70) <i>Streptococcus pneumoniae</i>	<i>Escherichia coli</i> <i>Pseudomonas aeruginosa</i> <i>Stenotroph. maltophilia</i>
Channel 706	Internal Control <i>Enterococcus faecium</i> <i>Enterococcus faecalis</i>	Internal Control

U. Reusch/IRMM/03.2007

Quantitative PCR is an issue in Diagnostic Microbiology but ...



U. Reusch/IRMM/03.2007

Sample Preparation Issues

... we usually encounter a broad spectrum of clinical specimens:

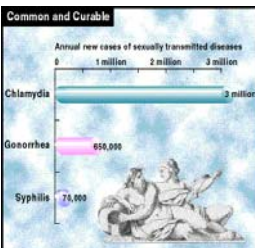
- Tissue (solid or soft tissue biopsies)
- Swabs (nasal swabs, wound swabs, rectal swabs)
- Blood (whole blood, blood culture, serum)
- Bone marrow aspirate
- Respiratory specimens (sputum, BAL, tracheal aspirate)
- Cerebrospinal fluid (CSF)
- Gastric juice aspirate
- Stool
- Urine
- Others ...



U. Reusch/IRMM/03.2007

Quantitative Real-time PCR for Chlamydia trachomatis

Common and Curable



Source: American Social Health Association

U. Reusch/IRMM/03.2007

CT-PCR Evaluation of Diagnostic Protocols in Clinical Bacteriology

- Chlamydia trachomatis -

Testing of MagNA Pure LC DNA preparations from urines and various kinds of swabs:

Total number of investigated samples > 1.200

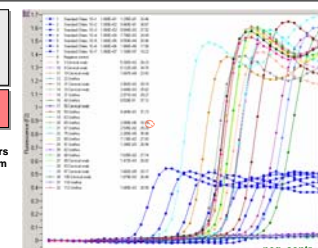
Clinical samples provided by Dr. Barbara Van der Pol

Swabs: 03-112

Premixed primers and probes (from TIB Molbiol)

T_m analysis: Internal Control (59°C, 64°C)

Quantitation (standard curve)



CT-WHO_02 (for Dr. Rosanna Pebody)

U. Reusch/IRMM/03.2007

Evaluation of Diagnostic Protocols in Clinical Bacteriology

- *H. pylori* - Clarithromycin resistance

Hybridization probe / melting curve-based detection of mutations in the *H. pylori* 23S rDNA gene associated with macrolide resistance:

PCR design according to: Maeda et al., 1998, *Gut* 43:317-321.

Evaluation of Diagnostic Protocols in Clinical Bacteriology

- *H. pylori* - Clarithromycin resistance

LC melting curve analysis performed with 30 cultured *H. pylori* isolates:

High Troughput Real-time PCR Testing

For screening purposes.

LC480

- 96-well
- 384-well
- 5 µl
- 20 µl
- 100 µl

Evaluation of Diagnostic Protocols in Clinical Bacteriology

- *lukFS* real-time PCR assay (LC 2.0) -

Screening of cultured *S. aureus* / MRSA strains for the presence of *lukFS*.

lukFS (PVL) in-house LightCycler assay (Regensburg)

Reference: Reischl et al., *CMID* (2007) 26:131-135

$T_m = 59^\circ\text{C}$ (P-*lukFS*)

T_m -analysis [F2]

Evaluation of Diagnostic Protocols in Clinical Bacteriology

- *lukFS* real-time PCR assay (LC 480) -

High throughput screening of *S. aureus* strains for the *lukFS* gene.

lukFS (PVL) in-house LightCycler assay (Regensburg)

Reference: Reischl et al., *CMID* (2007) 26:131-135

$T_m = 59^\circ\text{C}$ (P-*lukFS*)

T_m -analysis [F2]

Current and/or Intrinsic Limitations of Molecular Testing

or why NAT can only supplement but will not substitute diagnostic culture...

Limitations of Molecular Testing

Pre-PCR Processing ("Sample Preparation") still represents one of the central hurdles in molecular detection of bacterial pathogens

MOLECULAR BIOTECHNOLOGY Volume 26, 2004

REVIEW

Pre-PCR Processing

Strategies to Generate PCR-Compatible Samples

Peter Rådström,* Rickard Knutsson, Petra Wolffs, Maria Lövenkvist, and Charlotta Löfström

Category of Sample Preparation Method	Subcategory	Sample Preparation Method	Sample
Biochemical	Absorption	Lysozyme separation	Brief meat
		Protein adsorption	Blood
		DNA extraction	Hemolytic serum
Immunological	DNA extraction	Lytic methods	Blood anticoagulant
	Absorption	Immunomagnetic capture	Blood
Physical		Agarose two-phase system	Soft cheese
		Booyant density centrifugation	Miscel meat
		Concentration	Urine
		Dilution	Blood
		Filtration	Milk
		Enrichment	Meat

Pre-PCR Processing

→

Real-time PCR

U. Rådström/ITBM/03.2007

Potential Reasons for Discrepant Results between PCR and Culture

PCR: false-positive

- A positive culture result was observed with a previously or later examined specimen
- Mutations or deletions within a target gene, which may abolish the associated pathogenicity
- Cross-reactions with rare (apathogenic) variants of the corresponding pathogenic target organism

false-negative

- Mutations in popular target genes > pathogen may escape molecular detection (e.g. Swedish CT variant)
- Problem of quantity (relatively low counts of the corresponding pathogen in the investigated specimen)

~ 10² / PCR 1-5 / Culture

D.U. Rådström/ITBM/03.2007

Pathogen Distribution in the Starting Material

DNA extraction

200 µl

PCR

positive

DNA extraction

< 50 µl

PCR

false-negative!

➔ PCR is designed to find the "needle in a haystack" - and is usually able to do so...
...but the probability of finding this particular needle is dramatically reduced when only a small portion of the haystack is "analyzed"

U. Rådström/ITBM/03.2007

Near Future

High Resolution Melting

Rapid detection and precise characterization of point mutations or SNPs

C
(A) G G
↑ ↑
...g G A A a...

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Evaluation of Diagnostic Protocols in Clinical Bacteriology

- High Resolution (Amplicon) Melting Analysis -

Current diagnostic applications:
Homo / Heteroduplex analysis

Cystic fibrosis gene

Fig. 1. Genotyping the I507/F508 region of the cystic fibrosis gene with use of either SYBR Green I or LCGreen. (A), scaled drawing of the 44-bp amplicon. When SYBR Green I was used (B), the derivative melting curves showed closely spaced amplicon T_ms. No separate heteroduplex products were apparent. In contrast, when LCGreen was used (C), clearly separated heteroduplex products were observed that melted at lower temperatures than the homoduplex products. In both cases, two homozygotes and three heterozygotes were studied. The LightCycler instrument was used for melting curve acquisition.

Ref.: Wittwer et al. (2003) Clin. Chem. 49:853-860.

HRM-02 U. Rådström/ITBM/03.2007

Evaluation of Diagnostic Protocols in Clinical Bacteriology

- High Resolution (Amplicon) Melting Analysis -

Current applications of HRM:

The introduction of HRM has renewed interest in the utility of DNA melting, including:

- Mutation discovery (gene scanning)
- Screening for loss of heterozygosity
- DNA fingerprinting
- SNP genotyping
- DNA methylation analysis
- DNA mapping
- Species identification
- Somatic acquired mutation ratios
- HLA compatibility
- Identification of candidate predisposition genes

HRM-06 U. Rådström/ITBM/03.2007

Evaluation of Diagnostic Protocols in Clinical Bacteriology

- High Resolution (Amplicon) Melting Analysis -

Current diagnostic applications:
SNP analysis (HbS and HbC)

Ref.: Wittwer et al. (2003)
 Clin. Chem. 49:853-860.

Human β -globin gene

LC Green

Genotypes

- AA
- AS
- CC
- CS
- AC

Differences in melting kinetics of ds DNA

Temperature (°C)

Temperature (°C)

Fig. 3. Genotyping of two adjacent single-nucleotide polymorphisms (HbS and HbC) within a 110-bp β -globin fragment with use of high-resolution amplicon melting curve analysis.

The original high-resolution melting curves (A) include two different individuals for each of the six different genotypes, each individual run in duplicate. (B), the same curves as in panel A after normalization. (C), normalized curves after temperature-shifting of each curve to overlay them on one of the AA curves between 5% and 20% fluorescence. (D), fluorescence difference curves obtained by subtracting each curve in panel C from one wild-type (AA) curve (for clarity, the other AA samples are not shown). Because of the shift in temperature of the curves in panels C and D, the temperature axis no longer reflects absolute temperatures but rather reflects temperature differences relative to the superimposed segments of the curves.

HRM-03 U. Paetz/HRM/03.2007

Evaluation of Diagnostic Protocols in Clinical Bacteriology

- High Resolution (Amplicon) Melting Analysis -

Application: HRM for bacterial species differentiation (HR-1):

Clinical Chemistry 52:11
 1997-2004 (2006)

Rapid Detection and Identification of Clinically Important Bacteria by High-Resolution Melting Analysis after Broad-Range Ribosomal RNA Real-Time PCR

Ji-CHEN CHEN,¹ CHEN-LING FENG,^{1,2} CHEN-KE CHEN,^{1,3} CHI-CHANG CHEN,⁴ YA-CHIH CHANG,⁵ SHY-YIH CHANG,^{1,6,7} and CHENG-PING TSIANG^{1,5,6}

Temperature

Temperature

Fig. 3. Detection of bacteria by high-resolution melting analysis.

HRM-08 U. Paetz/HRM/08.2007

Evaluation of Diagnostic Protocols in Clinical Bacteriology

- High Resolution (Amplicon) Melting Analysis -

Current diagnostic applications:

Ref.: www.lightcycler480.com

LightCycler® 480 Real-Time PCR System

Example 3: Simultaneous genotyping of two adjacent SNPs (G51C/T, G63C/A) in the TNF- α gene promoter region.

A. Melting curves for different alleles and allele combinations, obtained with the depicted, internally labelled SimpleProbe probe.

Temperature (°C)

Temperature (°C)

Different allelic combinations of the investigated SNPs, illustrated by individual melting curves of samples with known genotype

HRM-04 U. Paetz/HRM/03.2007

Evaluation of Diagnostic Protocols in Clinical Bacteriology

- High Resolution (Amplicon) Melting Analysis -

Current diagnostic applications:

Ref.: www.lightcycler480.com

LightCycler® 480 Real-Time PCR System

Example 4: High-throughput haplotyping of adjacent SNPs by melting curve analysis with one HbProbe probe

Temperature (°C)

HRM-05 U. Paetz/HRM/03.2007

