The Potential of Multiplex Real-time PCR – A Modular Approach

Eduardo Thuroff, Toronto 2016
Agenda

• About TIB MOLBIOL
• Targets & Applications
• LightMix Kits
• **Multiplex PCR**
• PCR symmetry
• Fecal Panel
• ESBL, EHEC
• ModularDx Kits
• Outlook
Fluorescence is less sensitive when compared to radioactivity or enzyme-linked reactions.
Selection of Hybridization Probe Sequences for Use with the LightCycler

Offert Landt and Andreas Nitsche, TIB MOLBIOL, Berlin

1. Introduction

Detection of specific amplicons with hybridization probes provides real-time, sequence-specific analysis of amplified target sequences. For instance, this method can, during an amplification reaction, detect specific single copy sequences in genomic DNA or identify single-base mutations.

This Technical Note gives specific guidelines for selecting sequences that will make suitable hybridization probes.
Herpes simplex HSV-1/2

...and Quick Responders in Emergencies.
Typical Applications:

mRNA Quantification  
Pathogen Detections  
Pathogen Quantification  
Genotyping / Resistance

using

Hydrolysis Probes (TaqMan)  
Hybridization Probes  
SimpleProbe Oligomers (typing)

for carousel and plate based  
LightCycler® instruments.

design@tib-molbiol.de

Customized pre-mixes ('kits')  
and special release products (ASR) under ISO 13485 regulations
### Distribution of Real-Time PCR Applications

**Distribution of probe orders (custom designs and assays)**

<table>
<thead>
<tr>
<th>Category</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>pathogen detection</td>
<td>40%</td>
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<tr>
<td>clinical chemistry</td>
<td>15%</td>
</tr>
<tr>
<td>other disease related mutations, pharmacogenetics</td>
<td>10%</td>
</tr>
<tr>
<td>other SNP's</td>
<td>10%</td>
</tr>
<tr>
<td>cancer related quantification</td>
<td>10%</td>
</tr>
<tr>
<td>other mRNA quantification</td>
<td>10%</td>
</tr>
<tr>
<td>food, GVO, environmental</td>
<td>5%</td>
</tr>
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</table>

**~ 75% home brew human (medical) assays**
Distribution of Real-Time PCR Applications

Distribution of probe orders (custom designs and assays)

- 40% pathogen detection
- 15% clinical chemistry
- 10% other disease related mutations, pharmacogenomics
- 10% other SNP's
- 10% cancer related quantification
- 10% other mRNA quantification
- 5% food, GVO, environmental

~ 75% home brew human (medical) assays
# LightCycler® Dyes and Filters

The following channels are available in the various LightCycler® Instruments:

<table>
<thead>
<tr>
<th>Instrument</th>
<th>Channel ▶</th>
<th>530 F1</th>
<th>610</th>
<th>640 F2</th>
<th>&gt;660</th>
<th>670</th>
<th>700</th>
<th>705 F3</th>
<th>LC690</th>
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<tbody>
<tr>
<td>LightCycler® 1.2 / 1.5</td>
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<td>•</td>
<td>•</td>
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<td>(F3)</td>
</tr>
<tr>
<td>LightCycler® 2.0</td>
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<td>•</td>
<td>•</td>
<td>•</td>
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<td></td>
<td></td>
<td></td>
<td>(705)</td>
</tr>
<tr>
<td>LightCycler® 480</td>
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<td>•</td>
<td>•</td>
<td>•</td>
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<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>LightCycler® 480 II</td>
<td>•</td>
<td>•</td>
<td>•</td>
<td>•</td>
<td>•</td>
<td></td>
<td></td>
<td></td>
<td>(&gt;660)</td>
</tr>
<tr>
<td>LightCycler® Z480</td>
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<td>•</td>
<td>•</td>
<td>•</td>
<td>•</td>
<td></td>
<td></td>
<td></td>
<td>(700)</td>
</tr>
</tbody>
</table>

Typing: 530 + 640
Quantification: 640 + 690
LightMix® Duplex Typing 530/640

8. Sample data - typical results

Fig 1. Sample data for the human CYP2C19*2 and CYP2C19*3 detection system.

Upper panels: Data from channel 530. Left panel: melting peaks for human CYP2C19*2. Right panel: melting peaks for human CYP2C19*3. Wildtype (red) corresponds to CYP2C19*2; heterozygous (blue) corresponds to CYP2C19*2 and mutant (black) corresponds to CYP2C19*3.

Lower panels: Data from channel 640. Left panel: melting curves for human CYP2C19*3. Right panel: melting peaks for human CYP2C19*3. Wildtype (red) corresponds to CYP2C19*3; heterozygous (blue) corresponds to CYP2C19*2 and mutant (black) corresponds to CYP2C19*3.

Note: The values of the respective melting temperatures (Tm) may vary ± 1.5°C between different experiments. The ΔT between the melting peaks for heterozygous genotypes may vary ± 1.5°C.

Working with LightCycler® 1.0 and 2.0 and 480 systems.

LightMix® Kit Cytochrome P450 2C19 alleles *2 and *3
Replacement for discontinued Roche Diagnostics kit
LightMix® Duplex Quantification 640/690

Working with LightCycler® 1.x and 2.0 and 480 II systems.

LightMix® Kit Cytomegalovirus CMV with Internal Control
Why Multiplex PCR?

Similar clinical symptoms could be caused by a variety of pathogens, in particular in the fields of:

- Respiratory Infections
- Gastrointestinal Infections (diarrhea)
- Urogenital Infections (STI) (wetlands)

Multiplex testing (screening) on pathogens promises quicker answers and is more effective than single pathogen testing.
Agenda

- PCR Sensitivity & Specificity ?
- Multiplex TaqMan assay and PCR symmetry
- Hexaplex TaqMan assays Gastro
- Clinical Evaluation Data Gastro
- Hexaplex TaqMan assay ESBL
- Modular assays
- Extending the number of targets using the Nano
A primer with 17 nucleotides consents $4^{17}$ combinations:

$$4^{17} = 2^{34} \approx 4 \times 10^9$$

(should be unique in the human genome)
Primer Specificity: Norovirus

5'-AgCCAATgTTCAgATggATg Primer NV107a
     |           |
5'-AAAgggAgCgTAgATggATg Bacteroides (11/20 matches)

3'-gtgcagttgaggcaggcgg Bacteroides (upper strand)
     |     |     |
3'-gtgaat gaagatgccgtgc Primer NV119 (9/19 matches)
In a mixture of many primers we suspect multiple interactions between primer termini which can form primer dimers and so reducing the potential sensitivity, particularly in RT-PCR.

The complexity increases exponentially with number of components. Multiplex PCR can not be designed … (?)

We just tried …
Assay Normalization

- Primer Tm
- Lower limit: no product
- Higher specificity
- Optimum
- Limit temperature (no PCR product)

Laboratory standard condition

Temperature Ranges:
- 40°C
- 50°C
- 60°C
- 70°C
Simultaneous detection of five different DNA targets by real-time Taqman PCR using the Roche LightCycler480: Application in viral molecular diagnostics

Richard Molenkamp*, Alwin van der Ham, Janke Schinkel, Marcel Beld

Academic Medical Center, University of Amsterdam, Department of Medical Microbiology, Laboratory of Clinical Virology, P.O. Box 22008, 1100 DD Amsterdam, The Netherlands

Received 7 July 2006; received in revised form 30 October 2006; accepted 12 December 2006

and determines where this value is at its maximum. This value (crossing point, \( C_\text{t} \)) represents the cycle at which the increase of fluorescence is highest and where the logarithmic phase of a PCR begins. By using the second-derivative algorithm, data obtained are more reliable and reproducible, even if fluorescence is relatively low. The use of this second-derivative maximum algorithm might contribute greatly to the excellent qualitative

Acknowledgements

We would like to thank René Minnaar and Oliff Landi for stimulating discussions, Patricia Glasius for technical assistance and Nicholas Griffin for critically reading the manuscript.
Hexaplex TaqMan – Biosafety Pathogens

Combination of six assays – one target present only – sensitivity 10 copies
Burkholderia (YAK)

B. anthracis (FAM)

Brucella (Cyan500)

Coxiella (LC610)

Francisella (LC640)

Orthopox (LC670)

Hexaplex TaqMan – PCR symmetry?

sense primer dominant (20/5 pmol)
antisense primer dominant (5/20 pmol)
Symmetry studied by SimpleProbe melting

B. anthracis (30-9429-01) 10^6/10^4/10^2

anthr probe sense (1303468)

anthr probe antisense (1303469)

antisense primer dominant (5/20 pmol)
sense primer dominant (20/5 pmol)
Symmetry studied by SimpleProbe melting

B. anthracis (30-9429-01) $10^6/10^4/10^2$

- Anthr probe sense (1303468)
- Anthr probe antisense (1303469)

Francisella (30-8071-01) $10^6/10^4/10^2$

- Fran up (1304225)
- Fran down (1304226)

- Antisense primer dominant (5/20 pmol)
- Sense primer dominant (20/5 pmol)
Customer-specific Assay

**Hexaplex** Gastro Panel Bacteria and Parasites

LightCycler® 480
Hexaplex TaqMan - Gastro Panel (Bacteria)

Customer-demand:
- Combination of five different bacteria plus internal control
- Not 16S RNA as target because of unpredictable interactions

- High background of normal flora
  (E.coli, Enterococcus, Bacterioides, Lactobacillus, Klebsiella)
Hexaplex TaqMan - Gastro Panel (Bacteria)

Detection Limit < 10 copies (cloned standards)
Clinical Evaluation Bacterial Panel

Analysis of a total of 1281 fresh stool samples sent in for routine culture.

<table>
<thead>
<tr>
<th></th>
<th>Salmonella sp</th>
<th>Shigella/EIEC</th>
<th>Campylobacter</th>
<th>Y. enterocolitica</th>
<th>Plesiomonas</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture</td>
<td>pos</td>
<td>neg</td>
<td>pos</td>
<td>neg</td>
<td>pos</td>
</tr>
<tr>
<td>PCR-pos</td>
<td>13</td>
<td>5</td>
<td>2</td>
<td>4</td>
<td>19</td>
</tr>
<tr>
<td>PCR-neg</td>
<td>1</td>
<td>1262</td>
<td>0</td>
<td>1279</td>
<td>0</td>
</tr>
</tbody>
</table>

Campylobacter PCR was designed to detect diarrheagenic species (C. coli, C. jejuni, C. lari and C. upsaliensis) and not oral/commensal species such as C. fetis, C. mucoalis, C. spurotorum and C. ureolytica.
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<td>pos</td>
<td>neg</td>
<td>pos</td>
<td>neg</td>
<td>pos</td>
</tr>
<tr>
<td>PCR-pos</td>
<td>14</td>
<td>4</td>
<td>4</td>
<td>2</td>
<td>21</td>
</tr>
<tr>
<td>PCR-neg</td>
<td>0</td>
<td>1262</td>
<td>0</td>
<td>1279</td>
<td>0</td>
</tr>
<tr>
<td>Prevalence</td>
<td>1.1 %</td>
<td>0.3 %</td>
<td>1.6 %</td>
<td>0.3 %</td>
<td>0.2 %</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>100.0 %</td>
<td>100 %</td>
<td>100 %</td>
<td>75.0 %</td>
<td>100 %</td>
</tr>
<tr>
<td>Specificity</td>
<td>99.7 %</td>
<td>99.7 %</td>
<td>98.5 %</td>
<td>100 %</td>
<td>100 %</td>
</tr>
</tbody>
</table>
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<th>Y. enterocolitica</th>
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</tr>
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<tbody>
<tr>
<td>Culture pos/neg</td>
<td>pos/neg</td>
<td>pos/neg</td>
<td>pos/neg</td>
<td>pos/neg</td>
<td>pos/neg</td>
</tr>
<tr>
<td>PCR-pos</td>
<td>14/4</td>
<td>4/2</td>
<td>21/19</td>
<td>3/0</td>
<td>2/0</td>
</tr>
<tr>
<td>PCR-neg</td>
<td>0/1262</td>
<td>0/1279</td>
<td>0/1241</td>
<td>1/1277</td>
<td>0/1279</td>
</tr>
<tr>
<td>Prevalence</td>
<td>1.1%</td>
<td>0.3%</td>
<td>1.6%</td>
<td>0.3%</td>
<td>0.2%</td>
</tr>
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<td>100.0%</td>
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<td>98.5%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>PosPredVal</td>
<td>77.8%</td>
<td>50%</td>
<td>52.5%</td>
<td>100%</td>
<td>100%</td>
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<tr>
<td>NegPredVal</td>
<td>100.0%</td>
<td>100%</td>
<td>100%</td>
<td>99.9%</td>
<td>100%</td>
</tr>
</tbody>
</table>

Summary: Negative samples are probably true negatives. 70 / 1281 samples positive saves 1200 microbiology tests.
Parasitic Panel – First Samples

500 : Entamoeba
530 : Giardia
550 : Blastocystis
610 : Cryptosporidium
640 : PhHV
670 : Dientamoeba

Failure Color Compensation
Double infection
Color Compensation
Hexaplex TaqMan Parasitic Fecal Panel

Hans Kusters laboratory in Utrecht run a Clinical Evaluation 744 samples
Clinical Evaluation Parasitic Panel

744 sets of stool samples sent in for routine Triple Feces Test (TFT) and archival materials (fecal samples stored at -80°C).

<table>
<thead>
<tr>
<th></th>
<th>Cryptosporidium</th>
<th>Blastocystis</th>
<th>Giardia lamblia</th>
<th>Dientamoeba</th>
<th>Entamoeba his/dis</th>
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</thead>
<tbody>
<tr>
<td>Microscopy</td>
<td>pos</td>
<td>neg</td>
<td>pos</td>
<td>neg</td>
<td>pos</td>
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<tr>
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<td>14</td>
<td>0</td>
<td>129</td>
<td>33</td>
<td>20</td>
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<tr>
<td>PCR-neg</td>
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<td>740</td>
<td>3</td>
<td>579</td>
<td>1</td>
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<tr>
<td>Prevalence</td>
<td>1.9 %</td>
<td>17.7 %</td>
<td>2.9 %</td>
<td>9.2 %</td>
<td>4.0 %</td>
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<tr>
<td>Sensitivity</td>
<td>100 %</td>
<td>97.7 %</td>
<td>95.2 %</td>
<td>100 %</td>
<td>100 %</td>
</tr>
<tr>
<td>Specificity</td>
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<td>94.6 %</td>
<td>99.6 %</td>
<td>87.5 %</td>
<td>100 %</td>
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<tr>
<td>PPV</td>
<td>100 %</td>
<td>79.6 %</td>
<td>87.0 %</td>
<td>44.8 %</td>
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<td>NPV</td>
<td>100 %</td>
<td>99.5 %</td>
<td>99.9 %</td>
<td>100 %</td>
<td>100 %</td>
</tr>
</tbody>
</table>

Summary: Negative samples are probably true negatives.
ESBL

Oct 2012 inquiry for:
- NDM-1
- KPC
- Oxa48

Dec 2012 ::
- IMP
- VIM
- GES
Hexaplex TaqMan ESBL

VIM – Cyan500

NDM-1/2 - FAM

OXA-48 – R6G

KPC – LC610

IMP – LC640

Control – Cy5
## Gastro Assays

<table>
<thead>
<tr>
<th>LC480 II</th>
<th>Cyan500</th>
<th>FAM</th>
<th>HEX</th>
<th>ROX</th>
<th>LC640</th>
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</thead>
<tbody>
<tr>
<td>Gastro (Fecal Panel)</td>
<td>Pleisomonas</td>
<td>Campylobacter</td>
<td>Shigella</td>
<td>Salmonella</td>
<td>Yersinia</td>
</tr>
<tr>
<td></td>
<td>Aeromonas</td>
<td>Campylobacter</td>
<td>Shigella</td>
<td>Salmonella</td>
<td>Yersinia</td>
</tr>
<tr>
<td></td>
<td>Entamoeba</td>
<td>Giardia</td>
<td>Blastocystis</td>
<td>Cryptosporidia</td>
<td>Dientamoeba</td>
</tr>
<tr>
<td></td>
<td>E.histolytica</td>
<td>Giardia</td>
<td>Blastocystis</td>
<td>Cryptosporidia</td>
<td>Dientamoeba</td>
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</table>
# ESBL Assays

<table>
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<th>Cyan500</th>
<th>FAM</th>
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<tr>
<td>Gastro (Fecal Panel)</td>
<td></td>
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</tr>
<tr>
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<td>Cryptosporidium</td>
<td>Dientamoeba</td>
<td></td>
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<tr>
<td>E.histolytica</td>
<td>Giardia</td>
<td>Blastocystis</td>
<td>Cryptosporidium</td>
<td>Dientamoeba</td>
<td></td>
</tr>
<tr>
<td>ESBL 1</td>
<td>NDM-1/2</td>
<td>KPC</td>
<td>OXA48</td>
<td>IMP</td>
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<tr>
<td>ESBL 2</td>
<td>GES</td>
<td>IMP</td>
<td>VIM</td>
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</table>
## The Variety Problem – The Modular Solution

<table>
<thead>
<tr>
<th>LC480 II</th>
<th>Cyan500</th>
<th>FAM</th>
<th>HEX</th>
<th>ROX</th>
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</thead>
<tbody>
<tr>
<td>Gastro</td>
<td>Pleis.</td>
<td>Campyloba</td>
<td>Shigella</td>
<td>Salmonella</td>
<td>Yersinia</td>
</tr>
<tr>
<td>(Fecal Panel)</td>
<td>Aeromonas</td>
<td>Campyloba</td>
<td>Shigella</td>
<td>Salmonella</td>
<td>Yersinia</td>
</tr>
<tr>
<td>Entam.</td>
<td><em>Giardia</em></td>
<td>Giardia</td>
<td>Blastocystis</td>
<td>Cryptosporidia</td>
<td>Dientamoeba</td>
</tr>
<tr>
<td>E.hist.c.</td>
<td>Giardia</td>
<td>Giardia</td>
<td>Blastocystis</td>
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<td>Dientamoeba</td>
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<td>GES</td>
<td>IMP</td>
<td>VIM</td>
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<tr>
<td>Hexaplex</td>
<td>VIM</td>
<td>NDM-1/2</td>
<td>KPC</td>
<td>OXA48</td>
<td>IMP</td>
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</table>

The Dilemma: Laboratories have divergent target preferences.
## Modular Assays

<table>
<thead>
<tr>
<th></th>
<th>LC20,Nano</th>
<th>LC96, Z480</th>
<th>LC480</th>
<th></th>
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<tbody>
<tr>
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<td>530</td>
<td>580</td>
<td>530</td>
<td>580</td>
<td>610</td>
<td>640</td>
<td>660</td>
</tr>
<tr>
<td>Pleisomonas Entamoeba</td>
<td>Campylobacter</td>
<td>Shigella</td>
<td>Salmonella</td>
<td>Yersinia</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Giardia</td>
<td>Blastocystis</td>
<td>Cryptosporidia</td>
<td>Dientamoeba</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Norovirus 1,2</td>
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</tr>
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<td>Adenovirus</td>
<td>Rotavirus</td>
<td>Sapovirus</td>
<td>Astrovirus</td>
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<td>VIM</td>
<td>NDM-1/2</td>
<td>KPC</td>
<td>OXA48</td>
<td>IMP</td>
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<tr>
<td>Respiratory Virus</td>
<td>H1</td>
<td>InfA</td>
<td>InfB</td>
<td>PIV-1-4</td>
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<td>PIV-1</td>
<td>PIV-2</td>
<td>PIV-3</td>
<td>PIV-4</td>
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<td>HRV</td>
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<tr>
<td>Adenovirus</td>
<td>MPV</td>
<td>RSV</td>
<td></td>
<td></td>
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<tr>
<td>NL63</td>
<td>229E</td>
<td>OC43</td>
<td>HKU1</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Pneumonia</td>
<td>C.pn</td>
<td>M.pn</td>
<td>Legionella</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Herpes Virus</td>
<td>HSV-1</td>
<td>HSV-2</td>
<td>VZV</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>CMV</td>
<td>EBV</td>
<td>HHV-6</td>
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<td></td>
<td></td>
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<td></td>
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<tr>
<td>EHEC</td>
<td>Stx1</td>
<td>Stx2</td>
<td>Eae</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Newborn</td>
<td>TREC</td>
<td></td>
<td>KREC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TORCH</td>
<td>Rubella</td>
<td>CMV</td>
<td>HSV-2</td>
<td>Toxoplasma</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Internal Controls or Extraction Controls**
- Lambda or PhHV
- MSTN or Globin
- RNaseP or PhageMS
Modular Assays

Aeromonas single

Gastrobacteria

Aeromonas multiplex

Campylobacter single

Campylobacter multiplex
Modular Assays

Shigella single

Gastrobacteria

Shigella multiplex

Salmonella single

Salmonella multiplex
12. Multiplex PCR Compatibility

The VIM assay can be combined with other ESBL assays up to 8plex reactions including an internal control (IC) or desired extraction control (e.g., for PhoA) as depicted below:

<table>
<thead>
<tr>
<th>ESBL Multiplex PCR and Instrument Compatibility</th>
<th>PhoA</th>
<th>Z400</th>
<th>Z600</th>
<th>Z601</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC</td>
<td>Z400</td>
<td>Z600</td>
<td>Z601</td>
<td>PhoA</td>
</tr>
<tr>
<td>VIM</td>
<td>NEB</td>
<td>UKB1454</td>
<td>NEB</td>
<td>NEB</td>
</tr>
<tr>
<td>VIM</td>
<td>NEB</td>
<td>NEB</td>
<td>NEB</td>
<td>NEB</td>
</tr>
</tbody>
</table>

2. Storage at Arrival:

1. Multi orange 96-well VIM (lyophilized)
2. Multi black cap Positive Control (20,000 copies, lyophilized) - Do not freeze the lyophilized reagents

3. Storage and Stability:

- Lyophilized kits are stable for at least 6 months (2°C to 25°C) in the dark. See specific expiry date.
- Dissolved reagents are stable for at least 2 weeks if stored protected from light and cool (4°C).
- Dissolved reagents can be stored long-term at -20°C. Avoid multiple freeze-thaw cycles.

4. Additional Reagents Required

Roche LightCycler® 480 Probe Master Kit No. 04797494801

5. Introduction

Antibiotics containing a β-lactam ring like penicillins, cephalosporins and aztreonam are commonly used for treatment of a broad spectrum of Gram-negative as well as a few Gram-positive bacteria. β-lactamases are enzymes which can be produced by some bacteria and can result in the destruction of the antibiotic properties by opening the β-lactam ring of these antibiotics.

The Verona integron-encoded metallo-β-lactamase VIM, first reported in 1999 in Italy, is today widely distributed covering Europe, Far East, and the Americas. VIM occurs mostly in Pseudomonas (VIM-1 in X4K1252-1 pneumoniae), with more than 20 variants described.

6. Description

A 30 bp long fragment from the blaVIM gene is amplified with specific primers and detected with a hybridization probe with Cy5/500 label and dark quencher. The kit detects all blaVIM variants 1-36.

7. Specifications

- This assay detects 15 genuine equivalent copies or less per reaction (2-amplification dilution).

8. Sample Material and Extraction

- Typical samples are from feces or bacterial culture. See MolecularDi Diagnostics Extraction Protocol.

9. Material Safety Data Sheet (MSDS)

According to OSHA 29CFR1910.1209, Australia (NIOSHIC 1910, 1911, 1912) and the EU Directives 67/548/EEC and 1999/45/EC any products which do not contain more than 1% of a component classified as hazardous or classified as carcinogenic do not require a Material Safety Data Sheet (MSDS).

Product is not hazardous, not toxic, not IATF-controlled. Product is not from human, animal or plant origin. Product contains synthetic oligonucleotide primers and probes.

Certificate of Analysis (CoA)

[Signature]

Dr. V. Rehak

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CEO: Andreas Menzel (C5), Oliver Lauer (Regulär), Harald Börsch

20130501
8. Instructions for Use

- Instrument programming see document
- Color Compensation see instructions
- Pipetting instructions multiple PCR see

Modular DNA Programming DNA
48-6320 Universal Color Compensation NooMix
Module DNA Multiplex

8.1. Programming LightCycler® 480 Instruments

Use the instrument operators manual for details. Start programming before preparing the solutions.

The protocol consists of three program steps:

1. Denaturation: simple denaturation and enzyme activation
2. Cycling: PCRs amplification of the target DNA
3. Cooling: cooling the instrument

Default Format

LightCycler® 480 Instrument: 450-500
LightCycler® 480 II Instrument: 440-488
Cobas® 6800 Instrument (open channel): n/a
(see combination for Open50)

Table 1

<table>
<thead>
<tr>
<th>Program Stage</th>
<th>Denaturation</th>
<th>Cycling</th>
<th>Cooling</th>
</tr>
</thead>
<tbody>
<tr>
<td>Analyte Mode</td>
<td>None</td>
<td>Quantitative</td>
<td>None</td>
</tr>
<tr>
<td>Cycles</td>
<td>1</td>
<td>58</td>
<td>1</td>
</tr>
<tr>
<td>Target (C)</td>
<td>95</td>
<td>62</td>
<td>40</td>
</tr>
<tr>
<td>Hold (min-sec)</td>
<td>00:00:30</td>
<td>00:01:15</td>
<td>00:00:10</td>
</tr>
<tr>
<td>Pump Plate (C)</td>
<td>4.4</td>
<td>4.4</td>
<td>2.2</td>
</tr>
<tr>
<td>Pump Plate (M)</td>
<td>4.8</td>
<td>4.8</td>
<td>2.8</td>
</tr>
<tr>
<td>Acquisition</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
</tbody>
</table>


8.2. Experimental protocol

- Sample material: Use sequences nucleic acid preparations (e.g. High Pure Virus Nucleic Acid Kit, Roche)
- Negative control: Always test last one non-template control (NTC) - replace the template DNA with water.
- Positive control: Place a positive control - replace the template DNA with the provided control DNA.

8.2.1. Preparation of parameter-specific reagents (PCR, 96 reactions)

One reagent with a 'triple' cap contains all primers and probes to run 96 LightCycler® reactions.

Add 85 µl PCR-grade water to each reaction vial, mix the solution (vortex) and spin down.

For those vials containing the volume can be extended to 100 µl (signals will decrease by 10-20%).

Use 85 µl reagent for a 20 µl PCR reaction.

8.2.2. Preparation of the Positive Control DNA

Add 960 µl PCR-grade water to the vial with the 'DNA' cap. Mix by pipetting the solution up and down 10 times. Note: Opening of this vial may cause contamination of the work space (aerosol).

Use 5 µl positive control DNA for a 20 µl PCR reaction (7,600 copies / 5 µl).

8.2.3. Preparation of the Reaction Mix

In a cooled reaction tube, prepare the reaction mix: single reactions (left) or a single plate(right).

<table>
<thead>
<tr>
<th>One reaction</th>
<th>For with the Roche LC480 Probe Master</th>
<th>1/10 reactions</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.15 µl</td>
<td>Water, PCU-grade (strawberry cap, provided with the Roche Master Kit)</td>
<td>415 µl</td>
</tr>
<tr>
<td>0.25 µl</td>
<td>Reagent mix (vial contains reaction mix containing primers and probes)</td>
<td>25.5 µl</td>
</tr>
<tr>
<td></td>
<td>Control Reaction and additional compounds (Master PCR)</td>
<td>10.6 µl</td>
</tr>
<tr>
<td></td>
<td>Roche Master reaction cap (for preparation see Roche manual)</td>
<td>1.000 µl</td>
</tr>
<tr>
<td>15.6 µl</td>
<td>Volume of Reaction Mix</td>
<td>1,509 µl</td>
</tr>
</tbody>
</table>

Mix gently, spin down and transfer 15 µl per well.

Add 5 µl of this single or control DNA to each well for a final reaction volume of 20 µl. Seal plate and centrifuge.

9. Typical Results (Data from LightCycler® 480 II system)

10. Reading the Results

Perform data analysis as described in the operators manual. For multiple assays select the color compensation. We recommend using the Second Derivative Maximum method (automated 6° max).

View results in the 506 channel. The negative control (NTC) must show no signal.

Note: Cobas® 6800 instrument signal levels are about 59% compared to LightCycler® 480 II results.

11. References


TIB MOBILIO
High Throughput / Productivity
- adapted to run up to six dye channels
- DNA and RNA in one run
- protocols aligned to be run on the FLOW instrument line

Felixibility
- variable combinations possible
- customized assays (home-brew)
- can be applied on different instruments
<table>
<thead>
<tr>
<th>Category</th>
<th>Contents</th>
<th>Tested / Validated</th>
<th>IFU instructions to use</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primers &amp; Probes</td>
<td>Primer, TaqMan, HybProbe, SimpleProbe</td>
<td>No</td>
<td>No</td>
<td>Order by sequence, label and amount</td>
</tr>
<tr>
<td>Design Service</td>
<td>Primer, TaqMan, HybProbe, SimpleProbe</td>
<td>No evtl. publications</td>
<td>No published</td>
<td>Sequence proposals to end user</td>
</tr>
<tr>
<td>LightSNiP Assays</td>
<td>Premixed primers and SimpleProbe, no controls</td>
<td>Artificial targets + hu DNA on LC 480</td>
<td>Yes (short)</td>
<td>Pre-established and on-demand (then 2-3 weeks)</td>
</tr>
<tr>
<td>LightMix® Kits RUO/LSR</td>
<td>Premixed primers and probes. Includes Controls or Standards</td>
<td>Biological samples and controls as stated in manual</td>
<td>Yes (detailed)</td>
<td>Info on performance, amount of sample, sensitivity, specificity</td>
</tr>
<tr>
<td>LightMix® Kits CE-IVD</td>
<td>Primers and HybProbes, SimpleProbe. Includes Controls or Standards</td>
<td>Targets and human DNA, all LC stated in manual</td>
<td>Yes (detailed)</td>
<td>Specification of sample, purification method, study data / ref. method</td>
</tr>
<tr>
<td>ModularDx (FLOW)</td>
<td>Primer + TaqMan Probe</td>
<td>RUO or IVD</td>
<td>Yes</td>
<td>Gastro Hexaplex since 2010 Project started 2012</td>
</tr>
<tr>
<td>454 Plates</td>
<td>TP53, CEBPA, EZH, SFB3</td>
<td>Collaboration with Roche 454</td>
<td>Started 2012</td>
<td></td>
</tr>
</tbody>
</table>
A Winning Team

- More than 20,000 designs
- Over 1,200 LightSNIP Assays
- Over 100 LightMix® Assays
- Over 30 LightMix® Modular Assays

Whatever the challenge may be, put the strongest team on the field.
Doing now what Roche will need next