Multiplex-PCR in clinical virology
benefits and limitations

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Multiplex-PCR – the concept:
parallel amplification and/or detection
of different (viral) pathogens in one tube

potential advantages:
saving of
costs, time, technical resources, personnel
one set of primers for two viral targets: polyomaviruses JC and BK

system: quantitative real-time PCR in house-assay, ABI 7700
5'-exonuclease probes: JC-virus: FAM
BK-virus: VIC
one set of primer for both viruses
one set of primers for two viral targets: polyomaviruses JC and BK

advantage: same efficiency for both targets
disadvantage: strong interference

<table>
<thead>
<tr>
<th>Copies JC</th>
<th>1000</th>
<th>1000</th>
<th>1000</th>
<th>1000</th>
<th>1000</th>
<th>1000</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Ct for JC</td>
<td>32.8</td>
<td>33.4</td>
<td>31.5</td>
<td>33.9</td>
<td>32.5</td>
<td>36.5</td>
<td>Neg.</td>
</tr>
<tr>
<td>Ct for JC</td>
<td>33.1</td>
<td>31.8</td>
<td>31.2</td>
<td>33.3</td>
<td>31.1</td>
<td>32.6</td>
<td>Neg.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Copies BK</th>
<th>1000</th>
<th>1000</th>
<th>1000</th>
<th>1000</th>
<th>1000</th>
<th>1000</th>
<th>1000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ct for BK</td>
<td>32.2</td>
<td>32.9</td>
<td>33.5</td>
<td>33.8</td>
<td>32.6</td>
<td>36.8</td>
<td>Neg.</td>
</tr>
<tr>
<td>Ct for BK</td>
<td>33.5</td>
<td>33.1</td>
<td>33.4</td>
<td>34.2</td>
<td>33.1</td>
<td>37.2</td>
<td>39.9</td>
</tr>
</tbody>
</table>

Summary:

if the copy numbers of two viral targets differ more than 100- to 1000-fold, quantification will be not precise for the „low-copy-pathogen“ (but is this clinically relevant?).

in the case of presence of two or more viruses in a patient sample, one might miss pathogens if they are only present in low concentrations (“so what?”).

useful in settings with a very low percentage of patient samples containing two or more viruses.

will for example easily work for enteroviruses: coxsackie-, echo-, polio-, rhino-viruses
Inverness Medical ARGENE CMV, HHV-6,7,8 R-Gene™ Kit

CE-labeled assay for the detection of:

- Cytomegalovirus (quant.)
- Human Herpesvirus 6 (quant.)
- Human Herpesvirus 7 (qual.)
- Human Herpesvirus 8 (qual.)

ABI 7500 Fast Real-Time TaqMan-probes
Internal Control: VIC Viruses: FAM

Detection of CMV

CMV-standards
Detection of HHV-6

HHV-6 standards

CMV and HHV-6 linear regression

CMV

HHV-6
CMV-positive patient samples

CMV-positive patient sample
Patient sample plus human genomic DNA

Addition of DNA from human cells (10^5, 10^4, 10^3 cells) does not reduce the CMV-specific amplification signal.

Inverness Medical ARGENE CMV, HHV-6,7,8 R-Gene™ Kit

Summary:

- Specific and sensitive detection of CMV, HHV-6, HHV-7 and HHV-8 herpes viruses
- No cross-reaction between the different viral targets also at higher concentrations (data not shown).
- No detectable influence of “background”-DNA/RNA.
- Amplification of all four viral targets using one protocol for real-time detection, clear results, high throughput.
**Fast Track Diagnostic - Respiratory Pathogens**

Detects 15 different viral respiratory pathogens:

- Influenza A
- Influenza B
- RSV A
- RSV B
- Metapneumovirus A
- Metapneumovirus B
- Parainfluenza 1
- Parainfluenza 2
- Parainfluenza 3
- Parainfluenza 4
- Coronavirus 229
- Coronavirus 43
- Coronavirus 63
- Rhinovirus
- Adenovirus

24, 48 or 96 reactions / kit

**Platform:**
- ABI 7500 thermocycler
- Corbett Rotor-Gene 6000
- (or any other cycler which can detect FAM, VIC, YAK, CY5)

In addition needed:
- a real time PCR master mix and enzyme (recommended: AgPath-ID™ One-Step RT-PCR Kit, Ambion) + plastic consumables
- nucleic acid isolation system/kit (recommended for example easyMag, bioMerieux, Qiagen automated systems, but manual procedures also work)
Fast Track Diagnostic - Respiratory Pathogens

Setup: 5 primer/probe mixes and 2 pos. controls

- Yellow: mix for Flu-A, B and internal control (BMV)
- Red: mix for Cor63, Cor43, Cor229
- Light blue: mix for parainfluenza 2, 3, 4
- Brown: mix for metapneumovirus and parainfl.1
- Green: mix for rhinovirus, RSV-A, RSV-B, Adeno

- pos. control 1 (FluA-B, MPV, Para 1-4)
- pos. control 2 (rhino, RSV, corona, adeno)
Fast Track Diagnostic - Respiratory Pathogens

Respiratory Pathogens
all samples, all colors
Respiratory Pathogens
positive controls P1 and P2

positive control 1:
Flu-A/B, MPV, Para 1-4
range: Ct 27-33
fluorescence increase?
multicomponent view

positive control 2:
rhino, RSV, corona, adeno
range: Ct 27-33
fluorescence increase?
multicomponent view

Respiratory Pathogens
RSV-A/B as positive control

amplification view of
RSV-A/B in control P2

multicomponent view
of positive control P2
Respiratory Pathogens
Background

RSV-A/B
FAM channel

Adenovirus
CY5 channel

Flu-A/B separate and mixed
interference of different analytes

Respiratory Pathogens
Flu-A and Flu-B positive samples

Flu-A FAM

Flu-B CY5
Respiratory Pathogens
potential pitfalls / open questions

- Specific detection of 15 different viral respiratory pathogens
- Sensitivity remains to be analysed in detail (since expensive)
- Complex pipetting and analysis pattern (exper. report 7 pages)
- Pos. controls differ in concentration and fluorescence intensities
- How to proceed if positive controls are out of range?
- Detection of inhibition difficult (ICs show variable ampl. curves)

Summary

Benefits/Advantages:
- reduced amounts/input of expensive master-mix, probes
- many samples with short turn around time
- reduction in number of assay protocols, SOPs and detection platforms

Limitations/Problems:
- need for high amounts of viral nucleic acid
- interference of primers and probes, reduced sensitivity
- precise quantification can be a major problem
- complex analysis is error prone
Open questions in clinical virology

do you communicate a positive PCR result for a virus which was not included in your order?

do you communicate negative results for viral pathogens which were analysed but not requested?

How much do you charge (one extraction, one reverse transcription, one PCR or each of the analysed multiplex parameters)?

who is going to pay for 19 PCR-negative results?

which viral parameters should be combined in a multiplex assay – clinical relevance?

very much for your attention!

Thanks to the diagnostic PCR-team and especially to Anna-Lena Winkler and Helga Mairhofer!

... for allowing me to present their data - although with some personal consequences ...