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

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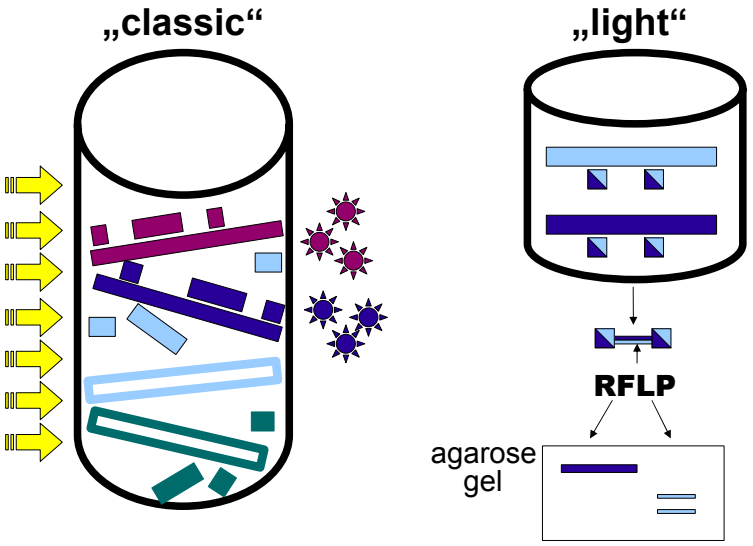
multiplex is not multiplex



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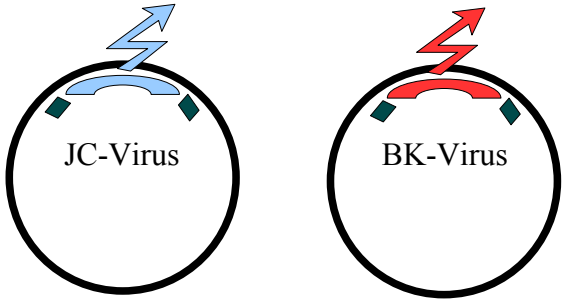
one set of primers for two viral targets: polyomaviruses JC and BK

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



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one set of primers for two viral targets: polyomaviruses JC and BK

advantage: same efficiency for both targets
disadvantage: strong interference

Copies JC	1000	1000	1000	1000	1000	1000	1000
Copies BK	0	10	100	1000	10 0000	100 000	1 000 000
Ct for JC	32,8	33,4	31,5	33,9	32,5	36,5	Neg.
Ct for JC	33,1	31,8	31,2	33,3	31,1	32,6	Neg.
Copies BK	1000	1000	1000	1000	1000	1000	1000
Copies JC	0	10	100	1000	10 000	100 000	1 000 000
Ct for BK	32,2	32,9	33,5	33,8	32,6	36,8	Neg.
Ct for BK	33,5	33,1	33,4	34,2	33,1	37,2	39,9

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one set of primers for two viral targets

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

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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

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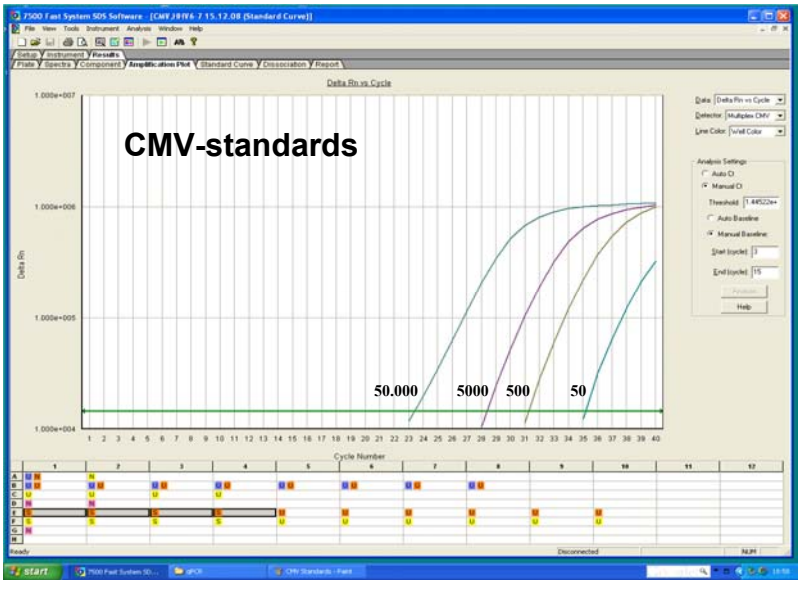
Detection of CMV



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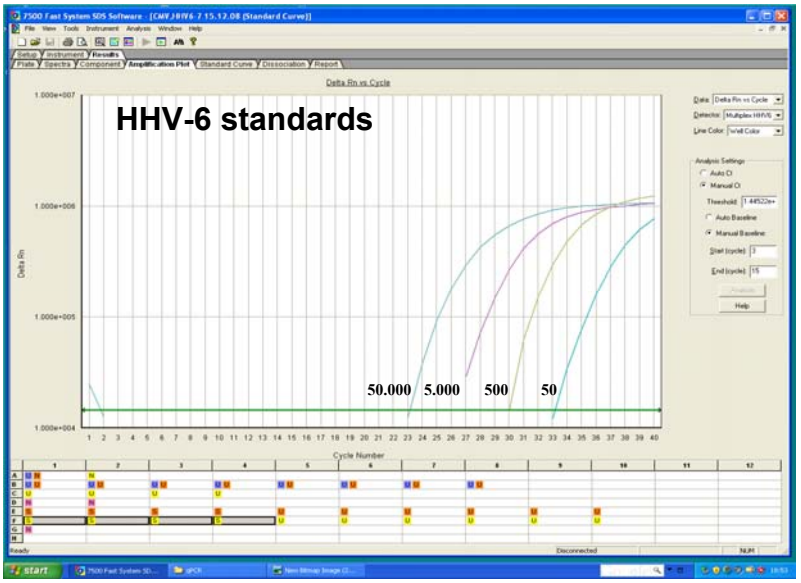
Detection of HHV-6



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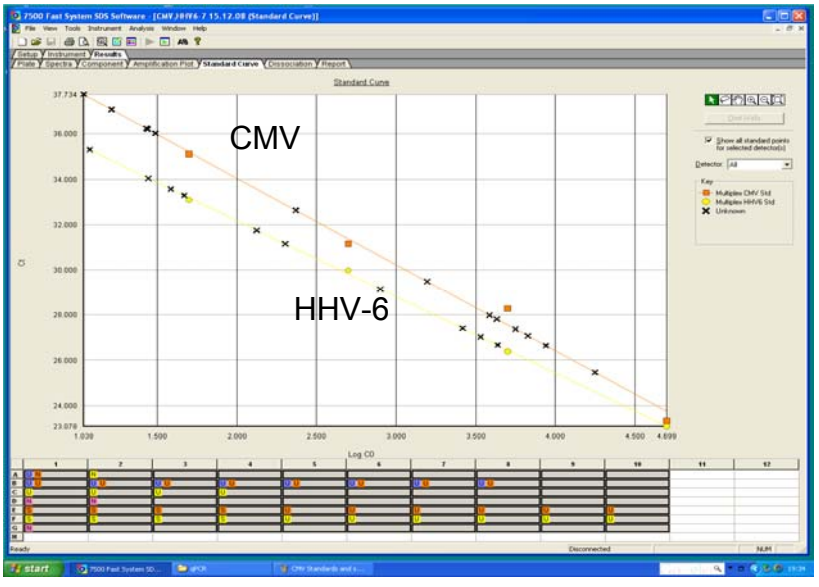
CMV and HHV-6 linear regression



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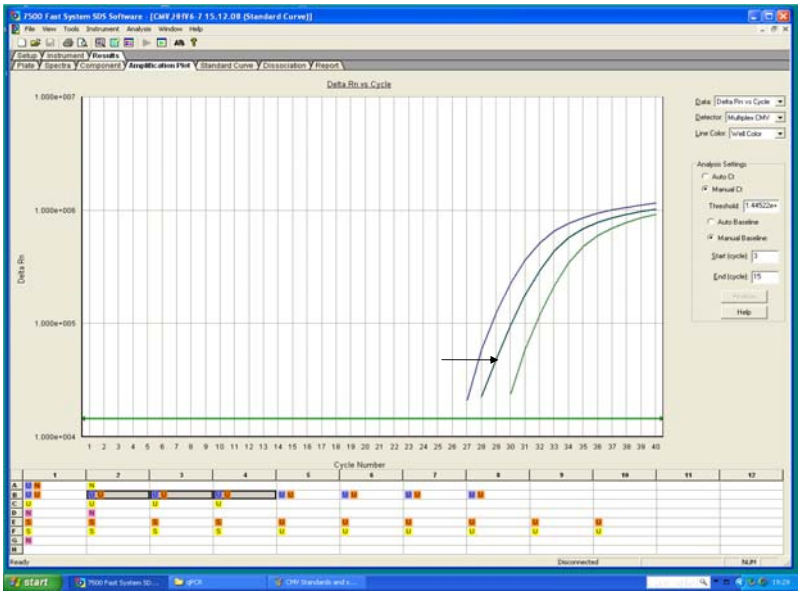
H N 2 0 0 9

CMV-positive patient samples



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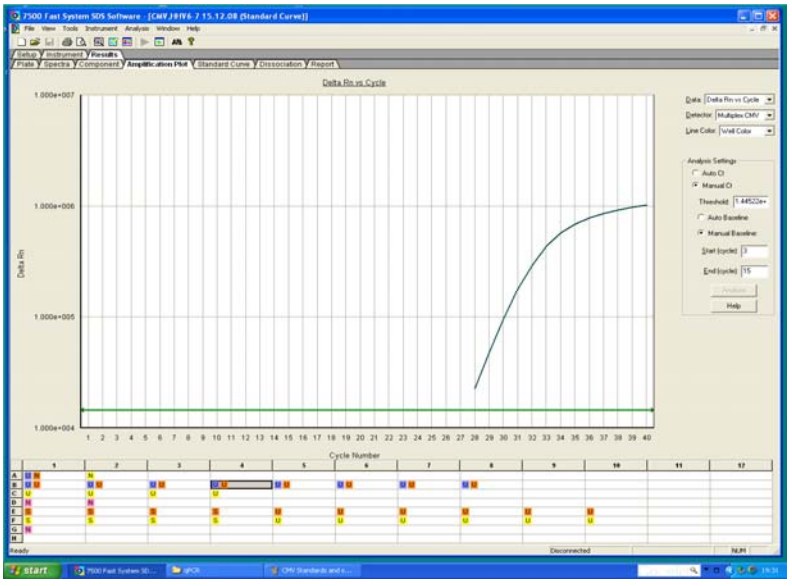
CMV-positive patient sample



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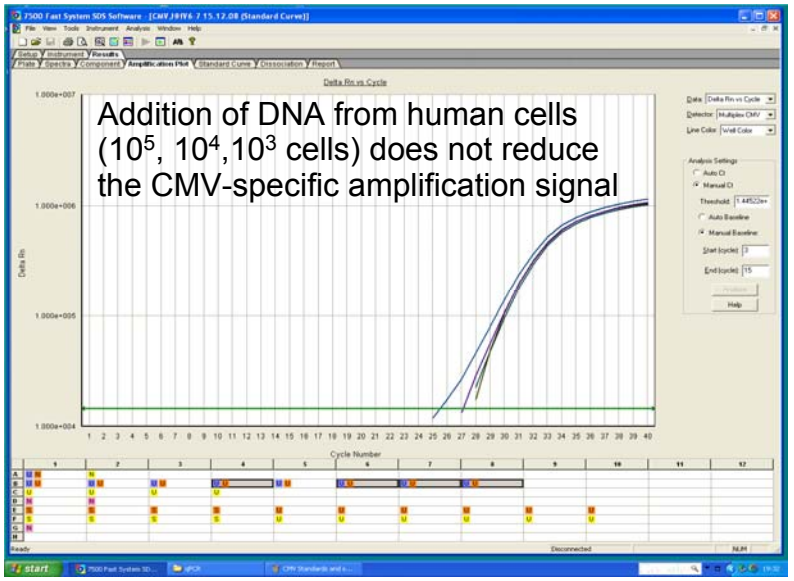
Patient sample plus human genomic DNA



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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.

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Fast Track Diagnostic - Respiratory Pathogens

Manual/protocol FTD-2-24/4

Detects 15 different viral respiratory pathogens:

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

24, 48 or 96 reactions / kit

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Fast Track Diagnostic - Respiratory Pathogens

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)

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Fast Track Diagnostic - Respiratory Pathogens

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

- mix for Flu-A, B and internal control (BMV)
- mix for Cor63, Cor43, Cor229
- mix for parainfluenza 2, 3, 4
- mix for metapneumovirus and parainfl.1
- mix for rhinovirus, RSV-A, RSV-B, Adeno

- pos. control 1 (FluA-B, MPV, Para 1-4)
- pos. control 2 (rhino, RSV, corona, adeno)

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Fast Track Diagnostic - Respiratory Pathogens

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

- FluA FAM, FluB CY5, BMV YAK
- Cor229 FAM, Cor63 YAK, Cor43 CY5
- Para2 VIC, Para3 FAM, Para4 CY5
- Para1 FAM, MPV-A/B CY5
- Rhino YAK, RSV-A/B FAM, Adeno CY5

- pos. control 1 (FluA-B, MPV, Para 1-4)
- pos. control 2 (rhino, RSV, corona, adeno)

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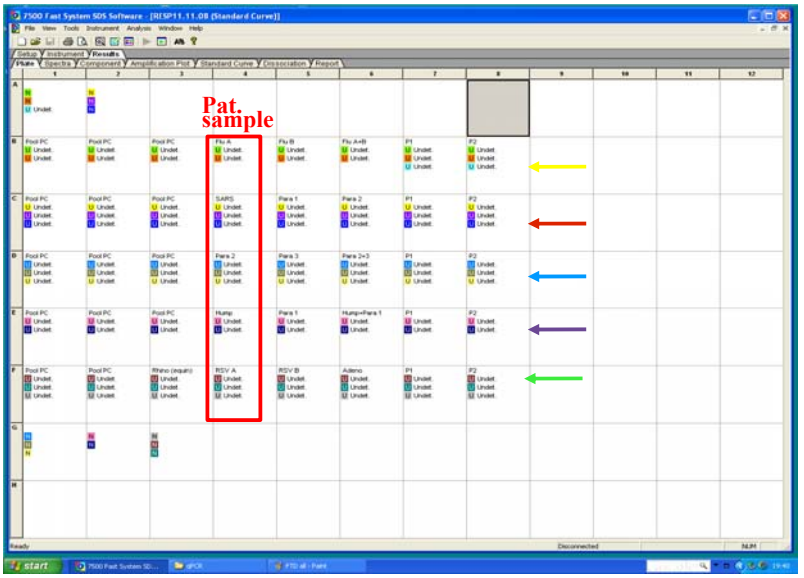
Fast Track Diagnostic - Respiratory Pathogens



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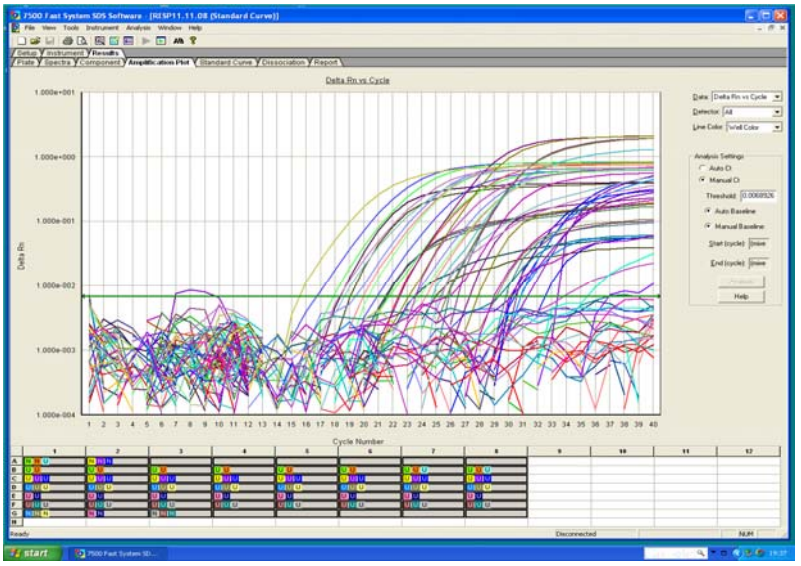
Respiratory Pathogens all samples, all colors



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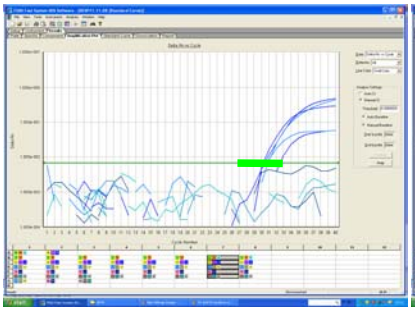
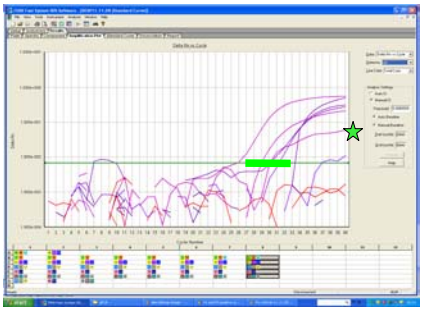
Respiratory Pathogens positive controls P1 and P2





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

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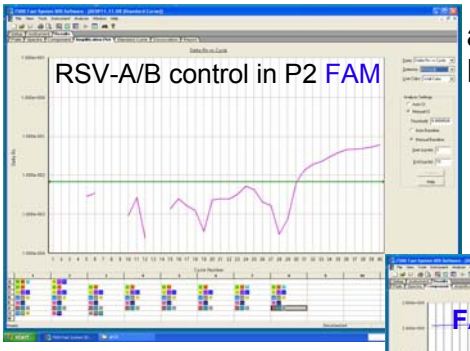
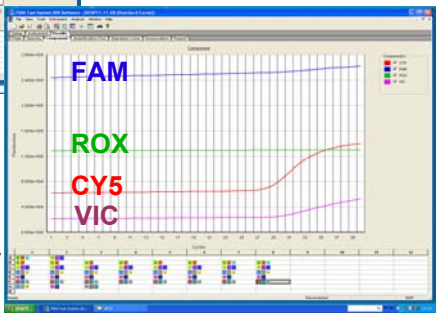
Respiratory Pathogens RSV-A/B as positive control



amplification view of RSV-A/B in control P2



multicomponent view of positive control P2

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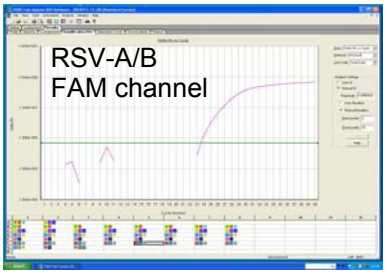
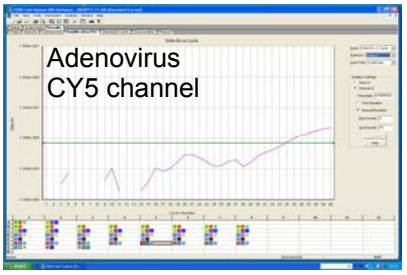
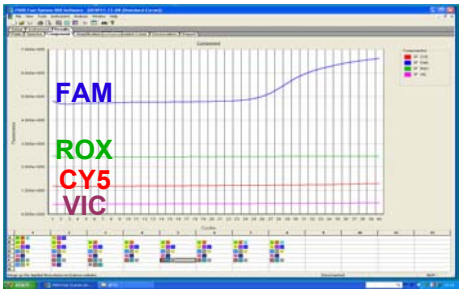
Respiratory Pathogens Background







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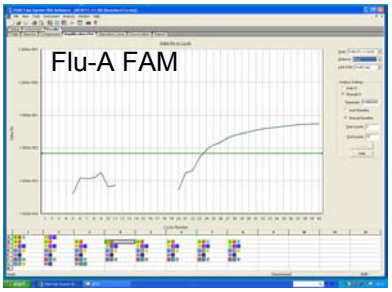
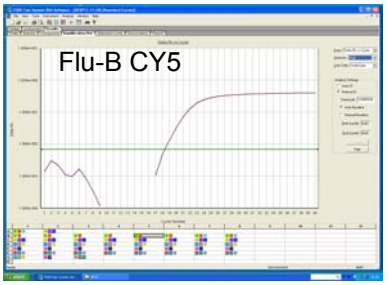
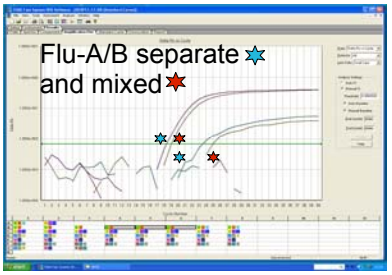
Respiratory Pathogens Flu-A and Flu-B positive samples







interference of different analytes

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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)

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Summary

Benefits/Advantages:	Limitations/Problems:
reduced amounts/input of expensive master-mix, probes	need for high amounts of viral nucleic acid
many samples with short turn around time	interference of primers and probes, reduced sensitivity
reduction in number of assay protocols, SOPs and detection platforms	precise quantification can be a major problem
	complex analysis is error prone

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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?

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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 ...