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Multiplex qPCR for influenza diagnostics

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Introduction

The German National Reference Centre for Influenza monitors the circulation of influenza viruses in Germany. Samples from patients presenting with influenza-like illness (ILI) are sent in for influenza virus diagnosis and differentiation of viral (sub)types (A/H1N1, A/H3N2, B). The diagnostic procedure includes the examination of samples by real-time PCR targeting different viral genome segments (M segment, HA segment, NA segment). To better cope with the high number of samples that arrive during the peak phase of the seasonal influenza wave, we aimed to speed up and simplify the diagnostic procedure by the introduction of a multiplex format for real-time PCR.

Methods

The PCRs were established with plasmid standards. All concentrations of the components were optimized for the conditions of multiplex PCR. The performance of the developed multiplex PCRs was compared to uniplex PCRs on the basis of influenza positive and negative specimens from the seasons 2006/2007 and 2007/2008. All samples were analyzed under the same PCR conditions. The PCR runs were performed with several real-time PCR systems (Applied Biosystems 7500, Stratagene Mx3000 and Mx3005, Roche LC 480).

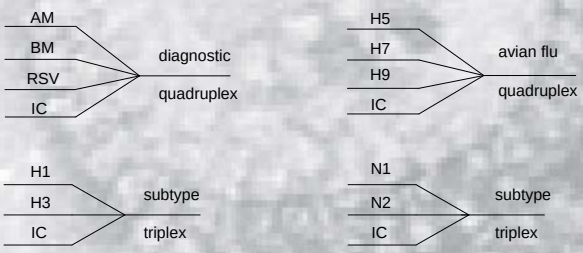

Figure 1: Multiplex real-time PCR assays (IC = internal control)

Results

Our uniplex PCR assays were combined according to diagnostic criteria (Fig. 1). In comparison to the uniplex approach, the quadruplex PCR comprising the assays for the detection of influenza A (AM), influenza B (BM), respiratory syncytial virus (RSV) and an internal control (IC) showed a comparable sensitivity and specificity (Fig. 2, Fig. 3). This was also observed for the quadruplex PCR H5/H7/H9/IC and the triplex PCRs H1/H3/IC and N1/N2/IC. Only negligible effects on the detection limit were observed for double infections. Although the C_t values remain unaltered for the multiplex assays, a reduction of fluorescence intensity was regularly observed. However, the reporter dyes FAM, VIC, Texas Red and Cy5 proved to be very well suitable as colour combination for quadruplex detection. To assure concordant results on miscellaneous qPCR platforms, the multiplex assays were tested on several instruments, and similar results were obtained.

After plasmid-based evaluation, the multiplex assays targeting human viruses were run on throat/nasal swab samples of patients with ILI from the 2006/07 and 2007/08 influenza seasons. These samples had been pre-tested positive and were re-examined with the uniplex as well as multiplex assays. The avian flu quadruplex was tested on virus isolates. 23 negative samples were also included into the panel to recognize false-positive results. As shown in Table 1, all samples were correctly identified for the AM/BM/RSV/IC and H1/H3/IC assays. However, the multiplex format missed one of 23 positive samples in the N1/N2/IC assay. The avian quadruplex PCR recognized all subtypes correctly. Both the AM/BM/RSV/IC and the H1/H3/IC assay are successfully applied for routine diagnostics in the current influenza season.

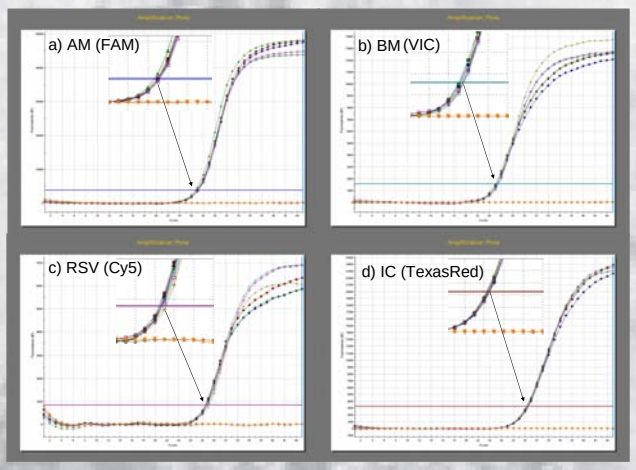

Figure 2a-d: Comparison of amplification plots:
A representative sample for each target was analyzed in triplicate with uniplex and multiplex PCR on the same PCR plate.

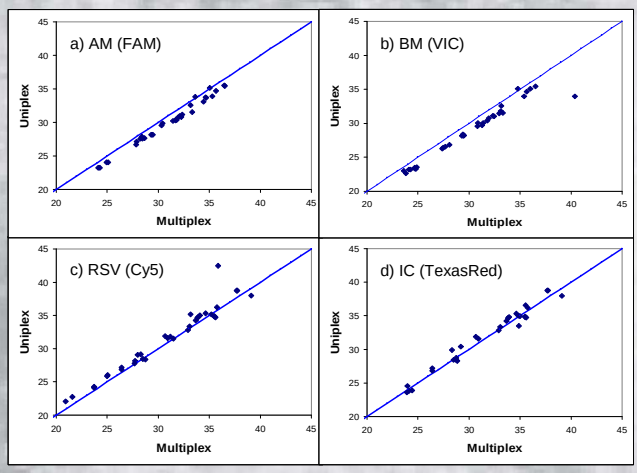

Figure 3a-d: Comparison of uniplex and quadruplex PCR C_t values:
A representative number of samples (Tab.1) was analyzed in parallel with uniplex and multiplex qPCR. Different concentrations of the internal control were also measured. The blue line indicates identical C_t values.

Conclusion

- Multiplex qPCR assays were established for the detection of:
  - Influenza A viruses, influenza B viruses, RSV + IC
  - Subtypes H1, H3 + IC
  - Subtypes N1, N2 + IC
  - Subtypes H5, H7, H9 + IC
- Specificity and detection limit for multiplex and uniplex PCR are comparable
- Similar results on several real-time PCR platforms such as
  - Stratagene Mx3000/Mx3005
  - Roche LC 480
  - Applied Biosystems 7500
- A significant reduction of hands-on time as well as financial costs is achieved

Table 1: Results of sample evaluation
A representative number of samples was analyzed in parallel with uniplex and multiplex qPCR. For the subtypes marked with * virus isolates were examined. See Figure 3 for the comparison of Ct-values.

	AM	BM	RSV	H1	H3	N1	N2	H5*	H7*	H9*	neg
correctly identified / total #	46/46	46/46	46/46	23/23	23/23	22/23	22/23	23/23	15/15	3/3	23/23

Multiplex qPCR is a powerful tool for the detection of influenza viruses