Development of a real-time PCR assay for quantitative analysis of human influenza A virus replication

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Introduction

The general course of influenza virus replication in host cells is well understood[1,2], but much about dynamics and control of viral genome expression as well as viral mRNA transcription still remains unclear. Here, we present the design and optimization of a sensitive and specific quantitative real-time PCR technique to investigate the time course of influenza A virus replication.

Principle of quantitation

We designed a two-step real-time PCR with SYBR green as fluorescence marker[3] (Fig.1). Infection was done in Madin-Darby canine kidney (MDCK) cells[4], with synchronization of infection by: a) use of high multiplicity of infection (MOI); b) 1h incubation at 4°C after virus addition; c) following washing step and medium exchange. Viral RNA was extracted from culture supernatant (extracellular) or from the cells (intracellular) using commercial RNA extraction kits.

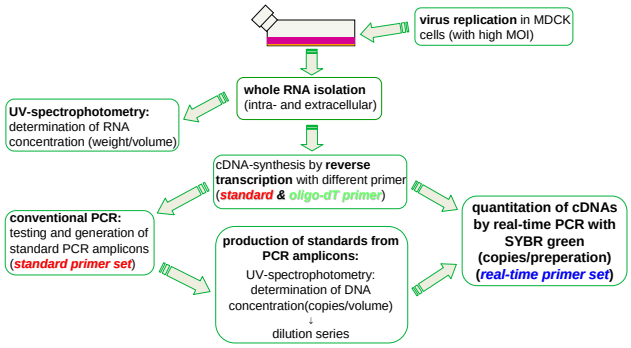

Figure 1: *Steps performed for quantitation of influenza viral RNA using real-time PCR*

Complementary DNA (cDNA) was synthesized in a reverse transcription step using different polarity specific primers (Fig.2): producing cDNA from either viral RNA of negative polarity (vRNA(-)) or from complementary RNA and messenger RNA of positive polarity (cRNA(+), vmRNA(+)) for influenza A virus.

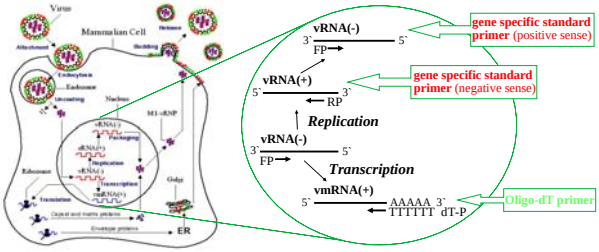

Figure 2: *Influenza virus A replication model[2]. Approach for polarity specific priming in reverse transcription for the synthesis of three different viral RNAs*

Experimental data

Primer design for segment 4 (HA) of human influenza A/PR/8/34 (H1N1) subtype, includes the creation of real-time primer used for real-time PCR & standard primer used for reverse transcription and production of standard PCR amplicons (Tab.1).

Table 1: *Designed primer for amplification of segment 4 (HA) viral RNA from influenza virus A/PR/8/34 (H1N1)*

Primer	Species	Gene	Sequence	Product length	Primer location
real-time for	A/PR/8/34 (H1N1)	HA (Seg4)	5` GGCCCAACCACAACACAAAC 3`	99 nt	451..470 nt
real-time rev			5` AGCCCTCCTTCTCCGTCAGC 3`		530..549 nt
standard for	A/PR/8/34 (H1N1)	HA (Seg4)	5` ACAGCCACAACGGAAAACTATG 3`	685 nt	187..208 nt
standard rev			5` CCGGACCCAAAGCCTCTAC 3`		853..871 nt

References

[1] Lamb RA, Choppin PW. *Ann Rev Biochem* **52** 1983, 467-506
[2] Sidorenko Y, Reichl U. *Biotechnology and Bioengineering* **88** (1) 2004, 1-14
[3] Arya M, Shergill IS, Williamson M, Gommersall L, Arya N, Patel HRH. *Expert Rev Mol Diagn* **5** (2) 2005, 209-219
[4] Genzel Y, Behrendt I, König S, Sann, H, Reichl U. *Vaccine* **22** (17-18) 2004, 2002-2008
[5] Mahy BWJ, Kangro HO. Academic press, London 1996, 41-43

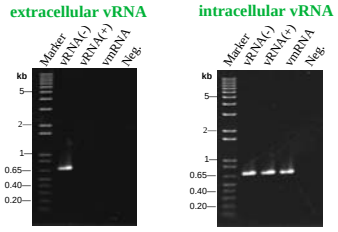

Figure 3: *Amplification of influenza virus segment 4 (HA) vRNA(-), vRNA(+) & vmRNA by PCR after reverse transcription step with polarity specific primer (Fig.2). Agarose gel electrophoresis & ethidium bromide. (Marker = DNA size marker (bp); Neg. = PCR negative control)*

The designed primer showed specific reverse transcription for different viral RNA types, synthesizing only cDNA from vRNA(-) with extracellular RNA but all viral RNA types with intracellular RNA (Fig.3). Specificity for segment 4 in PCR was determined by sequencing (data not shown). Because amplification efficiencies (E) of both primer sets are around 1, it can be considered as applicable for quantitation (Fig.4).

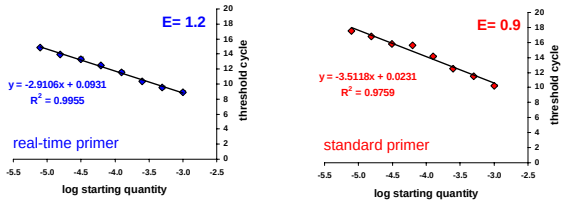

Figure 4: *Amplification efficiencies of segment 4 (HA) real-time & standard primer. Real-time PCR with both primer sets and diluted viral cDNA to generate calibration curves by plotting threshold cycle against corresponding dilution. Formula: $E = [10^{(-1/slope)}]-1$*

Similar values were found by validating four individually real-time standard curves (Fig.5):

- ❖ PCR efficiency: 1.1
- ❖ R^2: 0.97
- ❖ Sensitivity: 5 x 10^3 molecules
- ❖ STDEV: 3 – 6.5 %

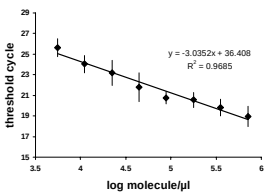

Figure 5: *Standard curve of segment 4 (HA) gene real-time PCR. Serial 2-fold dilution of segment 4 PCR-product (from 5 x 10^3 to 7 x 10^5 copies/µl) plotted against threshold cycle (mean of 4 replicate amplification of each dilution).*

Quantitation of a segment 4 time course of infection with extracellular RNA showed strong correlation to measured HA-titers (Fig.6). Gene specific priming seemed to be more sensitive than random priming in reverse transcription.

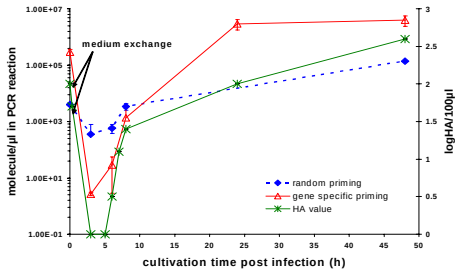

Figure 6: *Quantitation of extracellular segment 4 (HA) vRNA(-) with real-time PCR. Comparison of different priming strategies in reverse transcription against Hemagglutination assay (total amount of viral particles)[5]. (MDCK cells in GMEM infected with human influenza A (H1N1) with a MOI of 8)*

Outlook

- ❖ design and test of primer pairs specific for other segments
- ❖ quantitation of intracellular RNA (vRNA(-), vRNA(+), vmRNA)
- ❖ validation of the different steps of the quantitation method
- ❖ implementation into existing virus replication models[2]

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