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Detection of Avian H5N1 Virus with the LightCycler® Instrument

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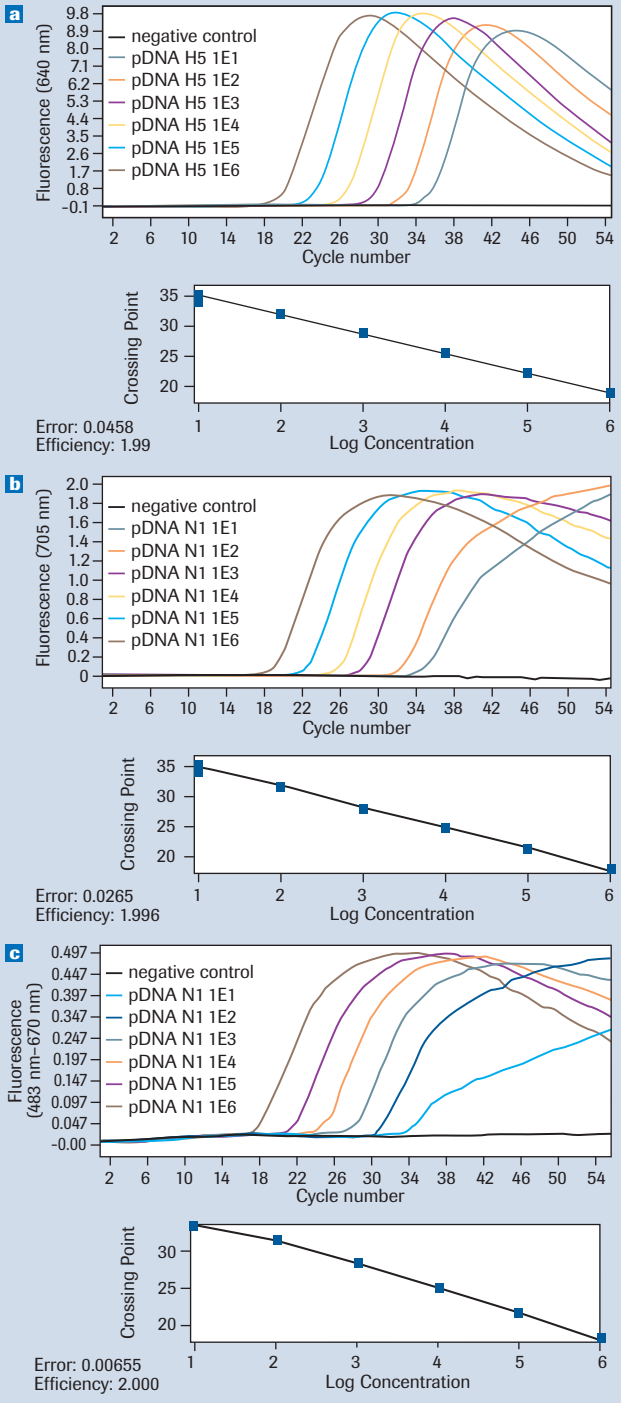

Figure 1: Amplification plots.
(a) Amplification plot for the H5 gene using the LightMix® kit H5N1 (Duplex PCR) and LightCycler® FastStart DNA Master HybProbe (channel F2 or 640nm).

(b) Amplification of the N1 gene (channel F3 or 705nm) using the LightCycler® 2.0 Instrument.

(c) Amplification with the LightCycler® 480 Instrument using LightCycler® Red 640 for the H5 gene (not shown) and LightCycler® Red 670 HybProbe probes for the N1 gene amplification.

Introduction

The avian H5N1 influenza virus has killed thousands of birds and caused several fatal cases in humans in Asia and Turkey. A fast and reliable test to identify the virus is urgently required for an effective epedemiological control, and associated medical research activities.

RNA viruses mutate easily. Thus, detection has to focus on conserved sequence regions. The preferred gene for the detection of a broad spectrum of influenza A virus variants is the matrix protein (M2) gene. Tests for the specific detection of the H5N1 virus must detect its hemagglutinin (H5) and neuraminidase (N1) genes.

Real-time PCR-based RNA virus tests using FRET hybridization probes are the method of choice. They also detect newly occurring variants as a specific signal in the melting curve analysis, even when a few bases at the binding site are exchanged.

Materials and Methods

To develop influenza A specific real-time PCR assays, a cDNA sample obtained from Thailand and derived cloned fragments were used. Tests were verified with a patient-derived sample from Hong Kong.

Using the Transcriptor First Strand cDNA Synthesis Kit, 10 µl of RNA were incubated for 30 minutes at room temperature to generate cDNA. 5 µl of cDNA were amplified using the LightCycler® FastStart DNA Master^{PLUS} HybProbe. A one-step RT-PCR protocol is available from the authors (currently in the process of optimization).

Results

We first demonstrated the accuracy and efficiency of the premixed LightMix® real-time PCR assay for H5 using the LightCycler® 1.2 Instrument during a conference held in Khon Kaen, Thailand, in October 2004.

Coincidentally, the PCR fragment amplified with this test was part of the amplicon recommended by the WHO in June 2005. The location of the sequence mutations in the

receptor-binding site of the H5 gene, recently reported in one isolate from Turkey, is included in this PCR fragment but does not overlap with primer or probe binding sites.

To duplex the H5 assay with an N1 specific target and achieve a sensitivity of at least ten genome copies (plasmid) for each targeted gene, we had to improve the H5 primers. The newly developed duplex assay was run with the LightCycler® 2.0 and LightCycler® 480 Instruments. In both cases the sensitivity was within ten copies (Figure 1).

The duplex H5N1 PCR was complemented with a PCR assay for the Matrix protein gene (M2) using the LightCycler® 2.0 Instrument, allowing detection of a broad range of influenza A virus types. We combined the probes from our earlier design [1] with published primers from the German Reference Laboratory [2] and adapted the primers to detect all new H5N1 isolates. The M2 PCR was completed by an Internal Control PCR based on the amplification of a fragment from Lambda DNA to verify the absence of PCR inhibitors.

All established primer systems have been combined with hydrolysis probes, sharing the binding sites of the HybProbe probes, yielding similar results in our experiments. In addition, we submitted some of the published H5 specific real-time PCR assays to a comparative test. In particular, the WHO-recommended primers derived from Yuen *et al.* [3, 4] performed well, whereas the hydrolysis probe assay from Spackman *et al.* [5] did not perform as expected. This was due to mismatches for the H5N1 target found in the forward primer H5+1456. Neither did the triplex hydrolysis probe assay from Payungporn *et al.* [6] give satisfying results in single PCR runs, and the triplex assay performed using the LightCycler® 480 Instrument was evaluated as inadequate. However, in contrast, both H5-specific hydrolysis probe assays from Ng *et al.* [7] (US CDC recommendation) showed a superior performance, reaching a sensitivity of fewer than ten genome copies in single-assay runs with the LightCycler® 2.0 Instrument and LightCycler® 480 Instrument (Figure 2). ■

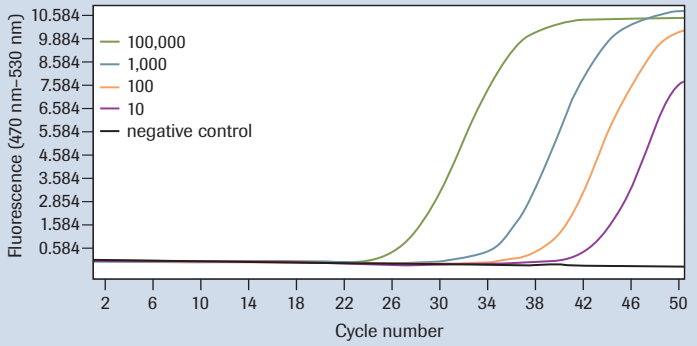

Figure 2: 5´-Nuclease assay performed using the TaqMan® probe H5-2P´ (FAM-TGGCAATCATGGTAGGCTGGTCTATCCTTATGG-BHQ1) [7] applied on a dilution row with 100,000, 1,000, 100, and 10 genome copies in the LightCycler® 480 Instrument.

References

1. Smith AB (2003) J Clin Virol 28: 51–58
2. Schweiger B (2000) JCM 38: 1552–1558
3. Recommended laboratory tests to identify avian influenza A virus in specimens from humans (2005) WHO Geneva
4. Yuen KY *et al.* (1998) Lancet 351: 467–471
5. Spackman E *et al.* (2003) JCM 40: 3256–3260
6. Payungporn S *et al.* (2005) Virol Methods
7. Ng EKO *et al.* (2005) Emerg Infect Dis

LightMix® products consist of premixed primers and hybridization probes for use with LightCycler® Instrument series. The HybProbe probe is lyophilized in 6 individual vials, 16 reactions each, providing a total of 96 reactions. Also included is a lyophilized standard row for quantitative analysis ranging from 10 to 10^6 target equivalents per reaction. The product does not contain Taq DNA polymerase or dNTPs. The product is designed for life science research only.

The LightMix® InfA kits have been tested by the German Reference Laboratories for Human Influenza (Robert-Koch-Institut) and for Veterinarian Influenza (Friedrich-Löffler-Institut) to be 'suitable for the detection of the AIV genome' and to detect all current H5N1 isolates.

Order INFO

Product	Pack Size	Cat. No.
LightCycler® FastStart DNA Master^{PLUS} HybProbe	1 kit (96 reactions)	03 515 575 001
Transcriptor First Strand cDNA Synthesis Kit	1 kit (50 reactions)	04 379 012 001
High Pure Viral Nucleic Acid Kit	1 kit (100 purifications)	11 858 874 001
LightCycler® 2.0 Instrument	1 instrument	03 531 414 201
LightCycler® 480 Instrument	96-well or 384-well instrument	04 640 268 001 04 545 885 001
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