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Timothy K W Cheung, Alex W H Chin, Kwok H Chan, Michael Schumaker, Polly W Y Mak, et al.. Evaluation of novel H1N1-specific primer-probe sets using commercial RT-PCR mixtures and a premixed reaction stored in a lyophilized format.. Journal of Virological Methods, Elsevier, 2010, 165 (2), pp.302-4. 10.1016/j.jviromet.2010.01.024 . pasteur-00588911

HAL Id: pasteur-00588911

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Submitted on 27 Apr 2011

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Accepted Manuscript

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PII: S0166-0934(10)00025-X
DOI: doi:10.1016/j.jviromet.2010.01.024
Reference: VIRMET 11134

To appear in: *Journal of Virological Methods*

Received date: 14-10-2009
Revised date: 27-1-2010
Accepted date: 28-1-2010

Please cite this article as: Cheung, T.K.W., Chin, A.W.H., Chan, K.H., Schumaker, M., Mak, P.W.Y., Leung, H.S.Y., Wong, A., Peiris, J.S.M., Petrauskene, O.V., Poon, L.L.M., Evaluation of novel H1N1-specific primer-probe sets using commercial RT-PCR mixtures and a premixed reaction stored in a lyophilized format, *Journal of Virological Methods* (2008), doi:10.1016/j.jviromet.2010.01.024

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Table 1. Performance of real-time RT-PCR for the novel H1N1 detection

RNA dilution factor (approximate TCID ₅₀)	HKU primer-probe set 1 ^a			HKU primer-probe set 2 ^a			CDC primer-probe set ^a			Lyophilized bead with HKU primer- probe set 2 ^a	CDC protocol ^{a,b}
	Kit 1 ^b	Kit 2 ^b	Kit 3 ^b	Kit 1 ^b	Kit 2 ^b	Kit 3 ^b	Kit 1 ^b	Kit 2 ^b	Kit 3 ^b		
1.0E+04 (1.68)											
No. of positive	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3
Mean CT	24.99	24.23	30.46	25.87	23.93	25.79	26.17	23.93	26.22	28.14	25.56
SD of CT	0.31	0.23	0.37	0.24	0.23	0.12	0.08	0.14	0.13	0.21	0.04
1.0E+05 (1.68E-01)											
No. of positive	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3
Mean CT	28.95	28.06	35.14	28.62	27.16	29.19	29.75	27.36	29.52	31.10	29.14
SD of CT	0.21	0.30	0.39	0.33	0.15	0.38	0.14	0.26	0.11	0.05	0.06
1.0E+06 (1.68E-02)											
No. of positive	3/3	3/3	0/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3
Mean CT	32.13	31.04	NA	31.23	30.30	32.29	33.04	31.00	32.92	34.00	32.54
SD of CT	0.65	0.32	NA	0.39	0.31	0.01	0.16	0.54	0.27	0.18	0.36
1.0E+07 (1.68E-03)											
No. of positive	3/3	3/3	0/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	2/3
Mean CT	35.70	35.26	NA	37.67	33.43	35.20	36.16	34.71	36.79	36.73	36.18
SD of CT	0.45	1.44	NA	1.41	0.42	0.35	1.14	1.23	1.10	1.14	NA
1.0E+08 (1.68E-04)											
No. of positive	0/3	0/3	0/3	0/3	2/3	1/3	2/3	1/3	1/3	1/3	1/3
Mean CT	NA	NA	NA	NA	36.40	38.03	37.53	35.95	38.16	35.83	38.25
SD of CT	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
1.0E+09 (1.68E-05)											
No. of positive	0/3	0/3	0/3	0/3	1/3	0/3	1/3	0/3	0/3	1/3	0/3
Mean CT	NA	NA	NA	NA	36.29	NA	34.93	NA	NA	35.69	NA
SD of CT	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
1.0E+10 (1.68E-06)											
No. of positive	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
Mean CT	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
SD of CT	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Water control											
No. of positive	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3

^a Primer and probe sequences:

HKU primer-probe 1 (Poon et al., 2009): Forward: 5'-GGGTAGCCCCATTGCAT-3', Reverse: 5'-AGAGTGATTACACTCTGGATTC-3', Probe: 5'-(FAM)TGGGTAAATGTAACATTGCTGGCTGG(TAMRA)-3'

HKU primer-probe 2 (Poon et al., 2009): Forward: 5'-GACAAGTTCATGGCCCAATCA-3', Reverse: 5'-TTTGCTCCAGCATGAGGACAT-3', Probe: 5'-(FAM)AACAAGGTGTAACGGCAG(MGB)-3'

CDC (Fraser et al., 2009): Forward: 5'-GTGCTATAAACACCAGCCTYCCA-3', Reverse: 5'-CGGGATTCCTTAATCCTGTRGC-3',

Probe: 5'-(FAM)CAGAATATACAT(BHQ1)CCRGTCACAATTGGARAA(Spacer 3)-3'

^b RT-PCR conditions:

Kit 1 (TaqMan EZ RT-PCR Kit):

- 4 µl RNA was amplified in a 25 µl reaction containing 2.5 U rTth DNA polymerase, 5 µl of 5x buffer A, 300 µM of dNTPs (except 1200 µM for dUTP), 3 mM of manganese acetate, 0.25 U of AmpErase UNG, 800 nM of forward primer, 800 nM of reverse primer and 400 nM of probe.

- 2 min at 50°C, 40 min at 60°C, 5 min at 95°C followed by 45 cycles of 95°C for 15 sec., 55°C (60°C for HKU primer-probe set 2) for 1 min.

Kit 2 (AgPath-ID One-Step RT-PCR Kit):

- 4 µl RNA was amplified in a 25 µl reaction containing 1 µl 25x RT-PCR enzyme mix, 12.5 µl 2x RT-PCR master mix reagent, 800 nM of forward primer, 800 nM of reverse primer and 400 nM of probe.

- 30 min at 50°C, 10 min at 95°C followed by 45 cycles of 95°C for 15 sec., 55°C (60°C for HKU primer-probe set 2) for 45 sec.

Kit 3 (RNA UltraSense One-Step Quantitative RT-PCR System):

- 4 µl RNA was amplified in a 25 µl reaction containing 1 µl 25x enzyme mix, 5 µl 5x reaction mix, 0.5 µl 50x ROX reference dye, 800 nM of forward primer, 800 nM of reverse primer and 400 nM of probe.

- 30 min at 50°C, 2 min at 95°C followed by 45 cycles of 95°C for 15 sec., 55°C (60°C for HKU primer-probe set 2) for 45 sec.

Lyophilized bead format:

- 30 min at 48°C, 2 min at 95°C followed by 45 cycles of 95°C for 15 sec., 60°C for 45 sec.

CDC protocol (SuperScript III Platinum One-Step Quantitative RT-PCR kit) (WHO, 2009c):

- 30 min at 50°C, 2 min at 95°C followed by 45 cycles of 95°C for 15 sec., 55°C (60°C for HKU primer-probe set 2) for 30 sec.

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Timothy K. W. Cheung¹, Alex W. H. Chin¹, Kwok H. Chan¹, Michael Schumaker², Polly W. Y. Mak¹, Horasis S. Y. Leung¹, Ada Wong², J. S. Malik Peiris^{1,3}, Olga V. Petrauskene², Leo L. M. Poon^{1*}

¹Department of Microbiology, The University of Hong Kong, Hong Kong Special Administrative Region, China

²R&D, Applied Biosystems, Foster City, California, USA.

³HKU-Pasteur Research Centre, Hong Kong Special Administrative Region, China

*Corresponding author: Dr Leo L. M. Poon
Department of Microbiology,
University Pathology Building,
Queen Mary Hospital, Pokfulam,
Hong Kong SAR,
China

Phone: 852 2819 9943

Fax: 852 2855 1241

E-mail address: lmpoon@hkucc.hku.hk

Abstract

The recent emergence of a novel H1N1 influenza A virus in humans caused the first influenza pandemic of this century. Many clinical diagnostic laboratories are overwhelmed by the testing demands related to the infection. Three novel H1N1-specific primer-probe sets reported during the early phase of the pandemic were tested in three commercial real-time RT-PCR mixtures. The amplification efficiencies and detection limits of these assays were determined. A ready-to-use premixed RT-PCR stored in a lyophilized format was developed. The detection limits of the studied assays were highly variable, ranging from 1.68E-01 to 1.68E-05 TCID₅₀ per reaction. The

detection limit of the lyophilized reaction mixture was found to be $1.68E-05$ TCID₅₀ per reaction, but the amplification efficiency of the assay was lower than those deduced from the other assays. All respiratory samples from infected patients and all control nasopharyngeal aspirates were positive and negative, respectively, in the newly developed assay. The results highlighted that, to enhance the sensitivity of an assay, it is essential to evaluate a primer-probe set with different commercial RT-PCR assays. This study also demonstrated the feasibility of using lyophilized reaction mixtures for the molecular diagnosis of novel H1N1.

Key words:

Influenza, Pandemic H1N1, Quantitative RT-PCR, Molecular diagnosis

Text

Influenza A virus belongs to the family *Orthomyxoviridae*. It is one of the viruses of medical importance. The unpredictability of this virus can be highlighted by the recent emergence of pandemic influenza A (H1N1) 2009 virus with genes derived from viruses that circulated in the swine, avian and human populations. The virus was identified initially in Mexico and the USA in March 2009 (Dawood et al., 2009; Fraser et al., 2009) and the disease has spread subsequently around the world within weeks. Several molecular diagnostic tests have been developed for detecting this novel human virus (Carr et al., 2009; Ellis et al., 2009; Fraser et al., 2009; Lau et al., 2009; Mahony et al., 2009; Poon et al., 2009; Wang et al., 2009). The latest update from the World Health Organization (WHO), issued on July 6 2009, reported 94,512 confirmed cases in 122 countries, with 429 deaths (WHO, 2009a). On July 16 2009, the World Health Organization announced that the number of laboratory-confirmed cases would no longer be counted (WHO, 2009b). This might be due partly to the fact that many diagnostic laboratories are overwhelmed by the considerable increase in the number of suspected cases and it might not be practical to confirm each of the suspected patients by laboratory diagnostic testing. Nonetheless, a close monitoring of unusual cases, such as patients with severe or fatal pandemic influenza A (H1N1) 2009 virus infection and suspected outbreaks in schools, are still recommended.

Several real-time quantitative RT-PCR assays for the novel H1N1 diagnosis were developed during the early phase of the pandemic (Fraser et al., 2009; Poon et al., 2009). In this study, some of these HA-specific assays were further optimized by testing

their primers-probe sets (HKU1, HKU2 and CDC, Table 1) in reactions derived from 3 different commercial quantitative RT-PCR kits. In addition, a ready-to-use lyophilized quantitative RT-PCR reagents for detecting the novel H1N1 was developed and evaluated.

Primer-probe sets developed from previous reports were evaluated in this study (Fraser et al., 2009; Poon et al., 2009). Viral RNA in culture supernatant of MDCK cells infected with A/California/04/2009 was extracted as described (Poon et al., 2009). Eluted RNA was stored in multiple aliquots at -80 °C until use. Ten-fold serially diluted RNA samples were prepared immediately before use. Diluted RNA samples were reverse transcribed and amplified by three different commercial one-step RT-PCR kits: Kit 1) TaqMan EZ RT-PCR Kit (Applied Biosystems, Foster City, USA), Kit 2) AgPath-ID One-Step RT-PCR Kit (Applied Biosystems, Foster City, USA) and Kit 3) RNA UltraSense One-Step Quantitative RT-PCR System (Invitrogen, San Diego, USA). The RNA samples were also tested by a RT-PCR assay recommended by the WHO as controls (WHO, 2009c). All reactions were analysed on a 7500 FAST Real-Time PCR System (Applied Biosystems). The primer-probe sets and amplification conditions of these real-time assays were summarized in Table 1. All the tests were done in triplicate.

As shown in Table 1, these commercial RT-PCR kits had different performances in detecting pandemic influenza A (H1N1) 2009 virus. With the same primer-probe set and RNA input, the cycle threshold (Ct) values obtained from the Kit 2 were generally smaller than those from the Kits 1 and 3. In contrast, except those reactions with the

HKU primer-probe set 1 (see below), the Ct values from the Kits 1 and 3 were comparable. Data obtained from the WHO-recommended protocol were included as references.

For the reactions with the primer-probe set 1, those from RT-PCR Kits 1 and 2 were found to have comparable results. But this primer-probe set performed poorly in reactions from the Kit 3 and the detection limit of this assay was at least 100 folds lower than those from the other two assays. For the reactions with the primer-probe set 2, those from the RT-PCR Kits 2 were shown to have the best performance. Of all the tests with the CDC primer-probe set, those using the RT-PCR Kit 1 were found to have the best sensitivity. Overall, our results demonstrated that the RT-PCR Kit 2 with the HKU primer-probe set 2 and the RT-PCR Kit 1 with the CDC primer-probe set are the best combinations in this study.

In order to reduce the enormous burden of laboratory diagnosis on clinical laboratories, attempts in simplifying the work procedures by developing a ready-to-use lyophilized quantitative RT-PCR bead were made. Reaction components, including buffer salts, ArrayScript reverse transcriptase (Applied Biosystems, Foster City, USA), AmpliTaq hot-start DNA polymerase (Applied Biosystems, Foster City, USA) and the primer-probe set 2 were mixed and lyophilized into a bead format. Reaction beads were manufactured by Applied Biosystems (Foster City, USA) upon a special request. Each lyophilized bead also contained a passive reference dye, ROX, for fluorescent signal normalization and an internal positive control (IPC) for PCR inhibitor detection. The IPC

probe was 5' labelled with VIC fluorescent dye and 3' conjugated with MGBNFQ quencher dye. The test consisted of lyophilized reactions organized into a ready-to-use 8-tube strip format. Lyophilized beads could be stored at 4 °C before use. These beads could be completely dissolved in water within 5 seconds. For a RT-PCR, a lyophilized bead was dissolved in 25 µl of RNase free water and 5 µl of RNA was added into the reaction mixture. The reactions were analysed by a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, USA) with the condition as indicated (Table 1). As shown in Table 1, the detection limit of the assay was comparable to those deduced from the most sensitive assays in this study ($1.68E-05$ TCID₅₀ per reaction). However, those reactions with a more concentrated RNA input (1.68 to $1.68E-02$ TCID₅₀ per reaction) were found to have a Ct value that is 2-4 cycles greater than those of the corresponding reactions from RT-PCR Kits 1-3. These results suggested that the reverse transcription and/or DNA amplification of this test might not be very efficient. All negative control samples (nasopharyngeal aspirate samples, N=30) and all respiratory samples from confirmed pandemic (H1N1) 2009 infected patients (N=29) were found to be negative and positive, respectively, in the test. All reactions were positive for the IPC.

In this study, the performances of 3 primer-probe sets in 3 different commercially available RT-PCR assays were compared. It was demonstrated that some of these combinations might have a better sensitivity and amplification efficiency. Nonetheless, some of these primer-probe sets were found to be performed poorly in some settings (e.g. HKU primer-probe set 1 in reaction from RT-PCR Kit 3). These results highlighted the importance of performing systematic assay optimizations.

The WHO recommended HA-specific real-time RT-PCR assay (WHO, 2009c), supplied by the USA CDC, was evaluated previously by others and the assay was found to be 10 folds less sensitive than other established real-time assays (Ellis et al., 2009). Similar results were obtained in the initial work of this study (data not shown). This might be due to the fact that the kit was only transported with chilled ice packs, suggesting the quality of the enzymes in the kit might be affected by the improper shipping method. Indeed, the sensitivity of the assay was found to be enhanced when a new batch of enzyme mixture was used in the work (Table 1).

In order to reduce the manpower and turnaround time for the molecular detection of pandemic influenza A (H1N1) 2009 virus, a ready-to-use premixed RT-PCR was converted into a lyophilized bead format. The presence of an internal positive control in the reaction could help to identify samples that might contain PCR inhibitors. In addition, it is expected that the simplified procedures might help to reduce human errors and PCR contaminations. In order to improve the sensitivity and amplification efficiency, the lyophilized reagents and the assay condition are currently under further optimization.

Acknowledgments

This work was supported by the Area of Excellence Scheme of the University Grants Committee Hong Kong Grant AoE/M-12/06 and the National Institutes of Health (NIAID contract HHSN266200700005C). We thank Brian Plew (Applied Biosystems) for technical support.

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