

## Development and Evaluation of a Real-time RT-PCR Technique for Detecting Matrix Gene of Influenza Virus Type A in Human Throat Swab Samples

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### Abstract

Influenza A virus is now considered to be widespread in poultry and has demonstrated the ability to infect humans in Iran. For laboratory diagnosis of these respiratory viruses, it is essential to have rapid methods, able to detect viruses in early stages of the infection in clinical specimens. The real-time reverse-transcription polymerase chain reaction (rRT-PCR) assay has been established as a standard method for Influenza virus type A diagnosis inpatients. In this study, we evaluated a single-tube rRT-PCR assay, targeting to the highly conserved region of matrix (M) gene for detection of the virus. In this experimental study, after preparation of 100throat mucus samples, respective RNA was extracted from the virus by using viral RNA extraction kit. Two specific primers were synthesized, based on the conserved region of Influenza type A gene and a home-brewed one-step SYBR Green based rRT-PCR was developed and evaluated for detection of Influenza type A infection in the viral samples, on the basis of melting curve analysis. The presence of M-gene in RNAs, extracted from 53viralsamples, was confirmed by this single-tube rRT-PCR assay, and after 45 amplification cycles, the melting curve analysis revealed the melting temperature ( $T_m$ ) of  $83.2 \pm 0.5^\circ\text{C}$  for various viral samples, quite different from that of primer-dimers and the positive samples showed only a small variation in parameters. This study showed that the developed one-step rRT-PCR assay is the proper molecular method for rapid and accurate diagnosis of Influenza A by detection of M-protein encoding gene.

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### Introduction

Influenza syndrome is defined by a rapid-onset systemic illness, with patients presenting with fever, chills, cough, myalgias, headache, and sore throat. Influenza A virus is a member of the family Orthomyxoviridae and can infect a wide diversity of animals as well as humans [1] although the ecological studies of these viruses have shown that wild aquatic birds are the original source of influenza A viruses [2]. The associated clinical symptoms in hosts and the result of influenza A virus infections considerably differ with the species of the host and strain of the virus. Influenza A viruses that are segmented negative strand genome viruses, classified according to the hemagglutinin (HA) and neuraminidase, the serological subtypes of the viral surface proteins (NA) [3, 4]. The HA gene of influenza virus was demonstrated to have a preferential association with the M (Matrix) gene [5, 6]. The M gene that is an important determinant of species specificity, encodes two partly overlapping proteins: a highly conserved 252-aminoacid M1 protein and a 97-amino-acid M2 protein [5, 7, 8]. Although the quotidian procedures for tracing of influenza A viruses described to date, including in vitro

virus isolation, immunofluorescence (IF), and the assays based on PCR, are powerful tools, they might be less effective for the diagnosis of influenza viruses with avian and swine source. The genetic and phenotypic heterogeneities of the latter viruses may cause a false-negative diagnosis of influenza A virus infection by in vitro cell culture or running protocols for analysis of PCR. Importantly, sporadic zoonotic events of influenza A virus infection can remain undetected as a result of such false-negative diagnoses [9]. The goal of this study was to evaluate a quick and accurate PCR method for screening of clinical samples for presence of genotypically and phenotypically various influenza A viruses. To this end, we developed a single-tube rRT-PCR assay panel, targeting to the highly conserved region of matrix (M) gene for detection of influenza A viruses, that was validated with clinical specimens.

### Materials and Methods

#### Sample preparation and viral RNA extraction

In this experimental study, after preparation of 100 throat mucus samples from suspected patients, viral RNAs were



extracted from 200  $\mu$ l of the virus-containing samples using a High Pure Viral RNA Extraction Kit (Roche-Germany) within 30 min, according to the manufacturer's instructions. Sample residues were stored at 4°C.

#### Synthesis of oligonucleotides

Specific primers (forward and reverse oligomers) targeting to influenza A virus M-gene (DQ321006.1), were adapted from WHO protocols and afterwards synthesized by Bioneer Company (Korea). The sequences of these specific primers, used for amplification and detection of influenza M-gene, have reported in Table 1. None of the primer sequences showed genomic cross-reactivity with other viruses, human genome and other probable interfering genomes in a BLAST search analysis and only detected a highly conserved 95 bp fragment of the matrix (M) region (results not presented).

**Table 1.** The sequences of specific primers used for amplification and detection of Flu A M-gene

Primer	Sequence	Product size (bp)
F	5'AAGACCAATCCTGTACCTCTGA-3'	95
R	5'-CAAAGCGTCTACGCTGCAGTCC-3'	

#### SYBR Green based single-tube rRT-PCR assay

The novel home-brewed SYBR Green based single-tube (one-step) rRT-PCR assay was developed using the QuantiFast SYBR Green RT-PCR Kit (Qiagen, Hilden, Germany) on Corbet (Rotor-Gene) 6000 (Qiagen, Germany) real-time PCR instruments. The amplifications were accomplished within 100 min in 20  $\mu$ l reaction mixtures containing 5  $\mu$ l of the target viral RNA and 2  $\mu$ l (20 pmol) of each primer (10  $\mu$ M). Cycling conditions were as follows: a single cycle of 10 min at 50°C, 5 min at 95°C; followed by 45 cycles of 10 sec at 95°C; and a final cycle of 30 sec at 60°C (analysis of fluorescence performed at the end of each 60°C step). So, the rRT-PCR products were detected via an increase in fluorescence from cycle to cycle. Also negative control or no template control (NTC) was used as the quality control of the process. Both cycling and melting curves in the real-time analysis were evaluated with respect to negative control and the test assay. The rRT-PCR amplicons were then confirmed by electrophoresis analysis.

#### Repeatability of the assay

All evaluations were performed with assay repeating by at least three different users and three times by each one of them in different days and the repeatability of the assays were confirmed.

#### Evaluation of analytical specificity by in silico prediction

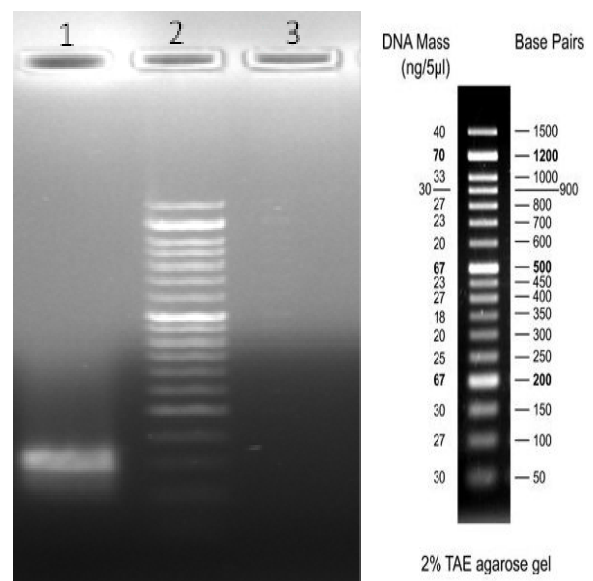
As previously mentioned, the highly conserved M-region sequence of influenza A virus genome was adapted from GenBank after alignment of the nucleotide sequences of available influenza A virus strains (obtained from NCBI database) and the specific primers targeting to this region were designed. None of the primer sequences showed genomic cross-reactivity with other viruses, human genome and other probable interfering genomes in a BLAST search analysis and only detected an about 100bp fragment of influenza A virus M-region (results not presented).

#### Evaluation of analytical sensitivity

In order to determinate the limit of detection (LoD), serial dilutions containing 3, 30, 300 and 3000 copies of positive control sample per reaction were analyzed in real-time PCR. The assay was evaluated by at least three different users and three times by each one of them in different days. The highest dilution of sample at which all replicates were positive was defined as the limit of detection (LoD) for this assay.

#### Results

Gel-based analysis of amplified fragment using the corresponding specific primers: After the amplification of influenza A virus M-region sequence by the specific primers, the expected ~ 100bp amplicon was confirmed by analysis of 2% electrophoresis gel (Fig. 1).

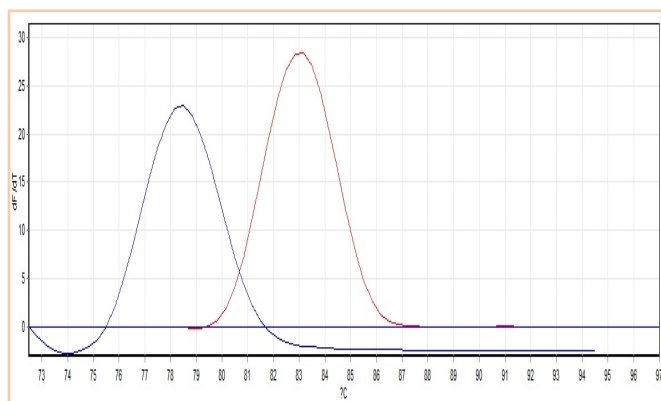


**Figure 1.** Electrophoresis analysis of influenza A virus M-region, amplified by gel based single-tube RT-PCR assay. Lane 1: PCR product with a length of ~ 100 bp fragment showing the positive result of assay, Lane 2: 50 bp DNA Ladder (Yekta-Tajhiz Azma, Iran), Lane 3: Negative control or NTC (No template control).

#### SYBR Green based one-step rRT-PCR assay

Melting curve analysis: In this novel home-brewed SYBR Green based single-tube rRT-PCR assay, the presence of M-gene in RNAs, extracted from 53 viral samples, was confirmed by this single-tube rRT-PCR assay and the products were identified based on  $T_m$  curve analysis and the PCR products from each primer-pair were generated based on individual  $T_m$  value. After 45 amplification cycles, the melting curve analysis revealed the melting temperature ( $T_m$ ) of  $83.2 \pm 0.5^\circ\text{C}$  for various viral samples, quite different from that of primer-dimers and the positive samples showed only a small variation in parameters.

So, only  $T_m$  peaks between  $83.2 \pm 0.5^\circ\text{C}$  can be acceptable as positive (observed in all tested samples) as shown in the melting curve in Figure 2 for one of these samples.



**Figure 2.** Melting curve analysis of one sample showed that melting temperature ( $T_m$ ) of specific amplicon was 83.2°C (red curve) and quite different from primer-dimers (blue curve).

### Analytical sensitivity of the assay

We prepared serial dilutions of positive control sample for evaluation of analytical sensitivity of the assay (results not presented). The serial dilutions were analyzed in real-time RT-PCR. The LoD of the assay signature was 30 or fewer copies of viral genomes /reaction (Table 2).

**Table 2.** Influenza A rRT-PCR assay limit of detection with positive control sample

Serial dilution (copies/reaction)	Positive testes(%) /M-region
3	42
30	100
300	100
3000	100

### Discussion

PCR based methods of virus diagnosis, have been described for many clinically related viruses. The sensitivity and specificity of these methods are determined by the choice of primer sequences. The sequences of the primers, described earlier for diagnosis of influenza A virus may be proper for detection of virus strains currently circulating in humans but show considerable numbers of mismatches when they are compared with the sequences of animal influenza A viruses [9]. In this study, we have used the specific primers (forward and reverse oligomers), targeting to influenza A virus M-gene, adapted from WHO protocols for diagnostic purposes in a wide variety of influenza A virus strains.

For rapid and accurate diagnosis, reverse-transcription polymerase chain reaction (RT-PCR) on extracted RNAs is a valuable technique [10]. Traditional (gel-based) RT-PCR methods require amplification in a thermo cycler (after cDNA preparation via RT process) and product analysis by electrophoresis gel, both of which are time-consuming and laborious [11]. By eliminating the need for post-amplification product processing, real-time RT-PCR method enables shortened turnaround times for reporting results, which is critical for deciding on suspected case patients isolation and contact tracing. Real-

time RT-PCR assays are sensitive and rapid and they can be automated. They have also greater reproducibility and since the need for post-PCR processing is eliminated, they can prevent carry-over contamination [11]. This technique not only provides confidence in identification of target genes, but also reduces the risk of facing with laboratory product contamination, that is because the amplification reaction and detection of PCR products are performed in a single tube [12].

Despite all advantages of the rRT-PCR assay, this technique is two-step. Step 1 is reverse transcription (RT) of RNA extracted from the virus, for cDNA preparation and step 2 is the real-time PCR. In this study, we developed a novel one-step rRT-PCR assay based on SYBR Green, instead of traditional two-step rRT-PCR assays, as the both of steps were out-and-out in single step in this assay that it can increase sensitivity of the assay and is affordable economically, in addition of other advantages, mentioned above.

### Conclusion

This research showed that the developed one-step rRT-PCR assay is the proper molecular method for rapid and accurate diagnosis of Influenza A virus by detection of a highly conserved region of matrix (M) gene.

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