PCR amplification of influenza A genomic segments for whole-genome sequencing

Introduction

Here we outline a workflow for amplifying all 8 influenza A genomic sequences for downstream Ion Torrent™ library preparation using the Invitrogen™ SuperScript™ III One-Step RT-PCR System with Platinum™ *Taq* High-Fidelity DNA Polymerase (Cat. No. 12574030). This workflow is an alternative to the workflow used with Applied Biosystems™ PathAmp™ FluA Reagents (Cat. No. 4485019), which was discontinued on July 31, 2016.

This workflow is based on testing done by scientists familiar with the PathAmp FluA Reagents workflow (**Pub. No. CO08519**) but unaffiliated with Thermo Fisher Scientific. The protocol is meant to serve as a guideline and has not been verified internally by Thermo Fisher Scientific.

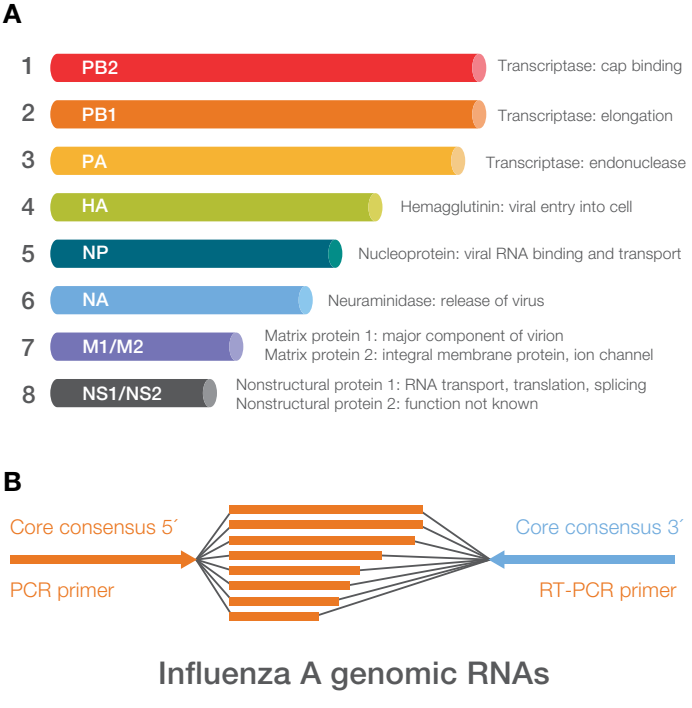

Figure 1. Primer design strategy for sequencing the influenza A genome. (A) Influenza A genome with 8 RNA fragments. **(B)** Primer design overview for the amplification of all 8 influenza A genomic segments.

Background

The influenza A genome is ~13.5 kb and comprises 8 segments that encode up to 11 proteins. The terminal segment-specific nucleotide regions are unique to each segment and are conserved across influenza A viruses, making it possible for researchers to design primers for reverse transcription and PCR amplification (Figure 1) and then to sequence the entire viral genome.

The SuperScript III One-Step RT-PCR System with Platinum *Taq* High-Fidelity DNA Polymerase is designed for sensitive, high-fidelity endpoint detection and analysis of RNA molecules by RT-PCR. Using this convenient one-step formulation, you can perform both cDNA synthesis and PCR amplification in a single tube using gene-specific primers and target RNA from either total RNA or mRNA. The system uses a mixture of Invitrogen™ SuperScript™ III Reverse Transcriptase and Platinum™ *Taq* DNA Polymerase High Fidelity for enhanced RT-PCR yields and fidelity, as well as the detection of longer templates. The system can detect a wide range of RNA targets from 300 bp to 10 kb, and is compatible with multiplex applications. The amount of starting material can range from 1 pg to 1 µg of total RNA.

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The primers used for this reaction (Table 1) were developed by Thermo Fisher Scientific to be highly specific universal primers for the amplification of all 8 influenza A genomic sequences, ranging in size from 900 bp to 2.4 kb.

Table 1. Materials used in the modified protocol.

Item	Cat. No.
Influenza A RNA template	Customer sourced
Primers Sense: 5′-CTGGATACGCCAGCRAAAGCAGG-3′ Antisense: 5′-GACCTGATGCGGAGTAGAAACAAGG-3′	Customer sourced
PCR-grade purified water	Customer sourced
0.2 mL nuclease-free, thin-walled PCR tubes	Customer sourced (e.g., MicroAmp tubes)
Real-time PCR thermal cycler	Customer sourced (e.g., QuantStudio system)
MagMAX-96 Viral RNA Isolation Kit*	AM1836
SuperScript III One-Step RT-PCR System with Platinum *Taq* High-Fidelity DNA Polymerase*	12574030
VetMAX-Plus Multiplex One-Step RT-PCR Kit*	4415330

* Protocol was tested with these products.

Protocol

1. Extract full-length influenza A viral RNA using the Applied Biosystems™ MagMAX™-96 Viral RNA Isolation Kit (Cat. No. AM1836) per the protocol (Pub. No. 1836M, Rev H).

2. Quantify RNA by RT-qPCR using the Applied Biosystems™ VetMAX™-Plus Multiplex One-Step RT-PCR Kit (Cat. No. 4415330) according to the protocol (Pub. No. 4426015, Rev C). Use a licensed PCR primer–TaqMan® probe mixture that is compatible with your real-time PCR system.

   See below for sequencing guidance based on RT-qPCR results; C_t values indicate the effect on sequencing when 8 µL of RNA is used in a total RT-PCR reaction of 25 µL.

C_t value*	Effect on sequencing
<30	Amplicons can be sequenced by the recommended protocol.
30–32	Amplicons may have decreased coverage and read depth, but should produce accurate sequencing results.
>32	Amplicons may be too low to titer for accurate sequencing using the recommended protocol. The number of amplification cycles may need to be increased.

* C_t value ranges were obtained using the Applied Biosystems™ VetMAX™-Gold SIV Detection Kit on extracted nucleic acid from porcine nasal swabs.

3. Amplify viral RNA with the SuperScript III One-Step RT-PCR System with Platinum *Taq* High-Fidelity DNA Polymerase (Cat. No. 12574030) per the protocol (Pub. No. MAN0001093, Rev A). See below for cycling conditions.

   i. **Programming the thermal cycler**
   The cycling parameters are set up so that cDNA synthesis is automatically followed by PCR amplification. This reaction will occur in three different phases: cDNA synthesis and denaturation, PCR amplification, and final extension.

   Note: Preheat the thermal cycler prior to setting up the reaction.

Phase	No. of cycles	Temperature (°C)	Duration
cDNA synthesis and denaturation	1	45	30 min
		95	1 min
PCR amplification	5	95	15 sec
		47	30 sec
		68	3 min
	23	95	15 sec
		57	30 sec
		68	3 min
Final extension	1	68	7 min

ii. **Setting up the reaction**

The final reaction mixture is 50 µL and contains reagents from the SuperScript III One-Step RT-PCR System with Platinum *Taq* High-Fidelity DNA Polymerase, as well as reagents provided by the customer from other sources. The reaction mixture should be combined in a 0.2 mL nuclease-free, thin-walled PCR tube held on ice for the duration of the reaction setup.

	Reagent	Volume (µL)
SuperScript III One-Step RT-PCR System with Platinum *Taq* High-Fidelity DNA Polymerase	2X master mix (containing 0.4 mM dNTPs, 2.4 mM $MgSO_4$)	25
	SuperScript III RT/ Platinum *Taq* High-Fidelity Enzyme Mix	1
Customer sourced	Template RNA	10
	Primer mix (5 µM mix of sense and antisense primers)	3
	Autoclaved distilled water	11
	Total reaction volume	**50**

Gently mix reagents. Make sure that all components are at the bottom of the tube prior to loading into the thermal cycler (centrifuge briefly if needed). Depending on the thermal cycler used, the protocol may require overlaying the reaction mixture with silicone oil.

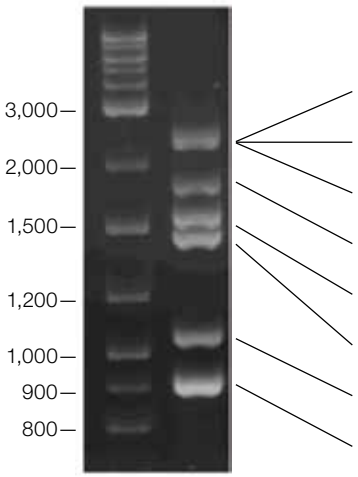
Place the reaction in the preheated thermal cycler programmed as described in the previous section, and run the program.

4. (Optional) Assess the integrity and size of the PCR products by gel electrophoresis (Figure 2).

5. (Optional) If electrophoresis of your sample shows RNA contamination, appearing as a diffuse smear at the bottom of the gel, treat the sample with RNase I and purify with the Invitrogen™ PureLink™ Genomic DNA Mini Kit (Cat. No. K182002) or equivalent, per the protocol (Pub. No. MAN0009847, Appendix C).

6. As the PCR products range in size from 900 bp to 2.4 kb, prepare libraries using the Ion Xpress™ Plus Fragment Library Kit (Cat. No. 4471269) per the protocol (Pub. No. MAN0009847, Rev F). Note: The protocol will depend on the chosen Ion Torrent™ platform (Ion S5™, Ion S5™ XL, Ion PGM™, or Ion Proton™ System) and sequencing read length.

7. Sequence libraries.



Segment	Gene	Length (bp)
1	Polymerase basic protein 2 (PB2)	2,341
2	Polymerase basic protein 1 (PB1)	2,341
3	Polymerase acidic protein (PA)	2,233
4	Hemagglutinin (HA)	1,777
5	Nucleoprotein (NP)	1,565
6	Neuraminidase (NA)	1,458
7	Matrix (M1/M2)	1,027
8	Nonstructural protein (NS1/NS2)	890

Figure 2. Agarose gel electrophoresis of influenza A RT-PCR amplification products. The 8 genomic segments have expected sizes of ~2,300, ~2,300, ~2,200, ~1,800, ~1,600, ~1,500, ~1,000, and ~900 bp. These segments should be visible as 6 bands, with the 3 longest genomic segments clustering together because they are not resolved on most agarose gels.

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