DEPARTMENT OF HEALTH & HUMAN SERVICES Public Health Service
Centers for Disease Control and Prevention (CDC)
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Multi-segment Reverse Transcription-PCR (MRT-PCR) of Influenza A and B Viruses

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1.0 Purpose

1.1 The purpose of this procedure is to describe a Single-Reaction Genomic Amplification of Influenza A and B viruses isolated from clinical specimens or grown viral isolates.

2.0 Definitions

2.1 MRT-PCR: Multi-segment Reverse Transcription-Polymerase Chain Reaction.

3.0 Critical Equipment

3.1 Microcentrifuge with strip tube adaptor

3.2 Tabletop centrifuge

3.3 Vortex

3.4 96-well format PCR Thermocycler System

3.5 Fragment Analyzer (must analyze fragments from 50 bp-5000 bp or more) (QIAxcel Advanced Instrument-Qiagen); (Fragment Analyzer™-Advanced Analytical Technologies, Inc.); or equivalent

3.5.1 **NOTE:** Can use gel electrophoresis system for PCR amplicons.

4.0 Laboratory Materials

4.1 96-well cold blocks for 0.2 ml and 1.5 ml PCR reaction tubes. (ISC BioExpress or equivalent).

4.2 Pipettes (2 µl, 10 µl, 20 µl, 200 µl, and 1000 µl)

4.3 Aerosol barrier pipette tips

4.4 0.2 ml PCR reaction tube strips or plates (examples below or equivalent)

4.4.1 PCR 8-tube strips, caps clear, (Brand Tech Scientific Inc. Catalog. No. 781332)

4.4.2 PCR Plate, 96-well, semi-skirted, flat deck. (Life Technologies, Catalog No. AB-1400)

4.5 2.0 mL self-standing conical screw cap microcentrifuge tube and cap with O-ring nuclease free and nonpyrogenic (Sarstedt: Catalog No. 72.694-006 or equivalent)

4.6 Sterile, nuclease free 1.5 ml micro-centrifuge tubes

4.7 GeneMate PCR sealing mats. (Bioexpress: Catalog No. T-3161-1 or equivalent)

4.8 TempPlate pierceable sealing foil, sterile. (USA Scientific: Catalog No. 2923-0110 or equivalent)

4.9 25 ml disposable reagent reservoir, sterile. (Axygen: Catalog No. RES-V-25-S or equivalent)

4.10 Sealing Roller. (BIO-RAD: Catalog No. MSR-0001 or equivalent)

4.11 Ultra-Pure Water (Molecular Biology Grade) Free of Detectable DNase, RNase and Protease

4.12 Invitrogen: SuperScript™ III One-Step RT-PCR System with Platinum™ Taq High Fidelity DNA Polymerase (Catalog No.: 12574030 (25 reactions), Catalog. No. 12574035 (100 reactions)

4.13 Gel Electrophoresis System and Supplies (if not using fragment analyzer for amplicons)

4.13.1 BenchTop pGEM® DNA Markers. (Promega: Catalog No.: G752A)

4.13.2 2% Agarose E-Gels with SYBR Safe DNA Gel Stain. Company. (Invitrogen: Catalog No. A421135) or equivalent.)

4.13.3 E-Base® electrophoresis system. (Invitrogen: Catalog No. G8100) with blue-light trans-illumination.

Universal primers for Influenza A and B listed in the following tables are from Integrated DNA Technologies Inc. (IDT) http://idtdna.com (or equivalent) **Oligos must be RNase Free HPLC purified

5.0 Universal INF Primers (For Influenza A) (Table I)

Oligo	# Bases	Sequence 5′-3′	Working Concentration	Amt (µl) from each 10 µM oligo to Pool
Uni12/Inf-1	18	GGG GGG AGC AAA AGC AGG	10 µM	20 µl
Uni12/Inf-3	18	GGG GGG AGC GAA AGC AGG	10 µM	30 µl
Uni13/Inf-1	22	CGG GTT ATT AGT AGA AAC AAG G	10 µM	50 µl

6.0 Universal Influenza B Virus-GA2 Primers (Table II)

Oligo	# Bases	Sequence 5′-3′	Working Concentration	Amt (µl) from each 10 µM oligo to Pool
B-PBs-UniF	20	GGG GGG AGC AGA AGC GGA GC	10 µM	75 µl
B-PBs-UniR	25	CCG GGT TAT TAG TAG AAA CAC GAG C	10 µM	75 µl
B-PA-UniF	20	GGG GGG AGC AGA AGC GGT GC	10 µM	50 µl
B-PA-UniR	25	CCG GGT TAT TAG TAG AAA CAC GTG C	10 µM	50 µl
B-HANA-UniF	20	GGG GGG AGC AGA AGC AGA GC	10 µM	60 µl
B-HANA-UniR	25	CCG GGT TAT TAG TAG TAA CAA GAG C	10 µM	60 µl
B-NP-UniF	20	GGG GGG AGC AGA AGC ACA GC	10 µM	75 µl
B-NP-UniR	25	CCG GGT TAT TAG TAG AAA CAA CAG C	10 µM	75 µl
B-M-Uni3F	23	GGG GGG AGC AGA AGC ACG CAC TT	10 µM	30 µl
B-Mg-Uni3F	23	GGG GGG AGC AGA AGC AGG CAC TT	10 µM	30 µl
B-M-Uni3R	28	CCG GGT TAT TAG TAG AAA CAA CGC ACT T	10 µM	60 µl
B-NS-Uni3F	23	GGG GGG AGC AGA AGC AGA GGA TT	10 µM	75 µl
B-NS-Uni3R	28	CCG GGT TAT TAG TAG TAA CAA GAG GAT T	10 µM	75 µl

7.0 Safety Precautions

7.1 Adhere to the safety guidelines provided in the Biosafety in Microbiological and Biomedical Laboratories and follow all established site-specific safety procedures, including wearing proper personal protective equipment (PPE).

8.0 Procedure

8.1 Assay Preparation

8.1.1 All reagents should be kept on a +4 to +8°C cold rack or ice during assay preparation.

8.2 Program the thermal cycler so that cDNA synthesis is followed immediately by PCR amplification:

- Step 1: Incubate, 42°C for 50 minutes
- Step 2: Incubate, 50°C for 10 minutes
- Step 3: Denature, 94°C for 2 minutes
- Step 4: Denature, 94°C for 30 seconds
- Step 5: Anneal, 43°C for 30 seconds
- Step 6: Extend, 68°C for 3 minutes, 50 seconds
- Step 7: Repeat steps 4-6 for 4 cycles
- Step 8: Denature, 94°C for 30 seconds
- Step 9: Anneal, 57°C for 30 seconds
- Step 10: Extend, 68°C for 3 minutes, 30 seconds**
    - **Extend this step by 10 seconds per cycle
- Step 11: Repeat steps 8-10 for 30 cycles
- Step 12: Final Extension, 68°C for 10 minutes
- Step 13: Hold, 4°C forever

9.0 MRT-PCR Procedure

9.1 Working in a uni-directional workflow, retrieve universal RT-PCR primers, SuperScript III One-Step RT-PCR kit, ultra-pure water, viral RNA, and influenza positive control RNAs from storage location and place on ice for thawing.

9.2 Vortex and briefly centrifuge all primers and place on ice.

9.3 Combine the components of the Superscript™ III One-Step RT-PCR System to prepare a reaction master mix (Table III and/or Table IV).

9.3.1 For multiple reactions, prepare a master mix with some excess reagent to allow for positive and negative controls and pipetting error. For example, for 96 samples, prepare a master mix for 110 samples (or 15% overage).

Master Mix for Influenza A viruses

Reagent	Volume (µl) Per Reaction
2X Rxn mix	12.5
Ultra-pure Water	8
SS III enzyme	0.5
INF Primer Pool	1
RNA	3
Total	**25**

Master Mix for Influenza B Viruses

Reagent	Volume (µl) Per Reaction
2X Rxn mix	12.5
Ultra-pure Water	7
SS III enzyme	0.5
B Primer Pool	2
RNA	3
Total	**25**

9.3.2 For 25 µl reaction volume, aliquot 22µl of the reaction mix into respective wells of a 96-well PCR plate or into 0.2-ml PCR tubes. Maintain all reactions on a cold block.

9.3.3 Add 3 µl of RNA from test samples and positive control reference viruses (H1N1, H3N2, and Bvic reference viruses). For no template controls (NTC), add 3 µl sterile nuclease free water.

9.3.4 Seal plate securely with sealing foil.

9.3.5 Briefly centrifuge to ensure that all the components are at the bottom of the amplification plate or tube.

9.3.6 Place the plate/tubes in the thermal cycler and run the MRT-PCR program (see 8.2).

10.0 Quality Control

10.1 MRT-PCR quality control analysis can be done with a fragment analyzer (must analyze fragments from 50 bp-5000 bp or more) such as with the Qiagen-QIAxcel Advanced Instrument or the Fragment Analyzer™-Advanced Analytical Technologies, Inc. (or any equivalent instrument).

10.1.1

10.2 Alternative: Analyze the MRT-PCR product by 2% agarose gel electrophoresis with SYBR Safe.

10.2.1 If using a precast E-gel (2% agarose with SYBR Safe), add 10µl distilled deionized H2O into each well using a multichannel pipette.

- Load 2-5 µl of MRT-PCR products including positive and negative controls into the respective wells of the E-gel. Load 10µl of DNA Marker into one or two unused wells and run the gel on the E-Base until appropriate separation occurs.
- Visualize the gel on a blue-light trans-illuminator (under an ethidium bromide filter) and save a picture of the gel.

Figure IA. RT-PCR products (INF primer set)

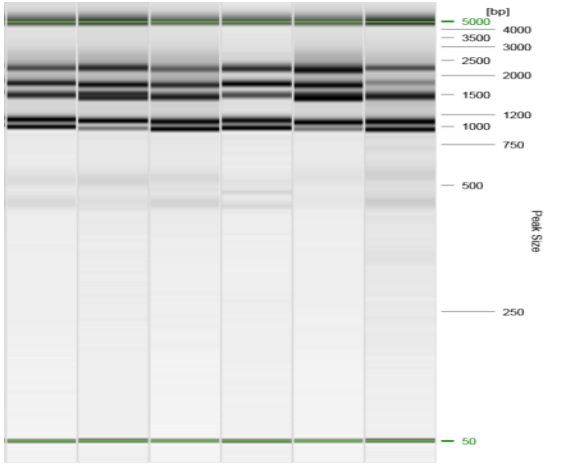
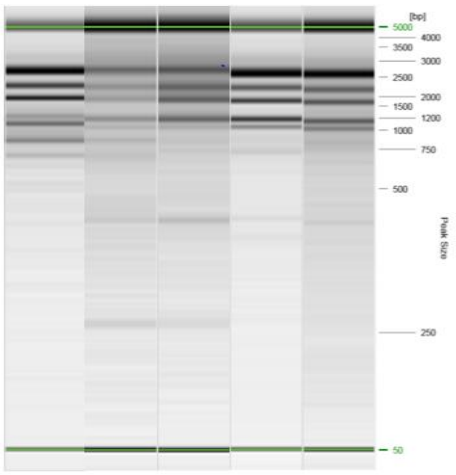


Figure IB. RT-PCR products (FLU B Virus-GA2 primer set)



11.0 Related Procedures

10.3 LP-381 – Automated RNA Extraction using the QIAcube HT

10.4 LP-347 – MRT-PCR Purification using Exonuclease I

10.5 LP-325 – QIAxcel Sample Preparation and Quality Control Procedure

11.0 Other SOPs and Documents

11.1 User's Guide for Superscript™ III One-Step RT-PCR System with Platinum® Taq High Fidelity. Company: Invitrogen

12.0 References

12.1 Zhou B, Donnelly ME, Scholes DT, St George K, Hatta M, Kawaoka Y, and Wentworth, D. Single reaction genomic amplification accelerates sequencing and vaccine production for classical and swine origin human influenza A viruses. J Virol. 2009;83:10309–13. 10.1128/JVI.01109-09

12.2 Zhou B, Lin X, Wang W, Halpin RA, Bera J, Stockwell TB, Barr, IG, and Wentworth, DE. Universal influenza B virus genomic amplification facilitates sequencing, diagnostics, and reverse genetics. J Clin Microbiol. 2014;52(5):1330–7.