



Multi-segment Reverse Transcription-PCR (MRT-PCR) of Influenza A Viruses for Sequencing

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1.0 Purpose

- 1.1 The purpose of this procedure is to describe a single-reaction multi-segment amplification of influenza A viruses.

2.0 Definitions

- 2.1 MRT-PCR: multi-segment reverse transcription polymerase chain reaction
- 2.2 Room temperature: 15-25°C

3.0 Equipment and Materials (Use Examples or Equivalent)

- 3.1 Sterile, nuclease-free 1.5 mL micro-centrifuge tubes
- 3.2 0.2 mL PCR reaction tube strips or plates
 - 3.2.1 PCR 8-tube strips (Brand Tech Scientific Inc. Catalog. No. 781332)
 - 3.2.2 PCR Plate, 96-well, semi-skirted, flat deck (Life Technologies, Catalog No. AB-1400)
 - 3.2.3 TempPlate pierceable sealing foil, sterile (USA Scientific: Catalog No. 2923-0110)
 - 3.2.4 Sealing Roller (BIO-RAD: Catalog No. MSR-0001)
 - 3.2.5 Silicone compression mat (Sigma Aldrich: Catalog No. AXCYCMFLAT)
- 3.3 Vortex
- 3.4 1.5 mL tube, 0.2 mL strip tube, and 96 well plate compatible centrifuge
- 3.5 Cold blocks for 0.2 mL and 1.5 mL PCR reaction tubes (ISC BioExpress)
- 3.6 Pipettes (10 µL, 20 µL, 200 µL, and 1000 µL)
 - 3.6.1 Multichannel pipettes also recommended (20 µL, and 200 µL)
 - 3.6.2 Corresponding aerosol barrier pipette tips
- 3.7 Disposable reagent reservoir, sterile (Axygen: Catalog No. RES-V-25-S)
- 3.8 96-well format PCR Thermocycler System (BioRad: T100)
- 3.9 1.5 mL tube, 8-tube strip, or 96-well format magnetic separation rack (Life Technologies DynaMag-2: Catalog No. 12321D, Alpaqua Magnum FLX: Catalog No. A000400)
- 3.10 DNA electrophoresis and visualization equipment (QIAxcel advanced: Catalog No. 9001941)



4.0 Reagents

- 4.1 Nuclease-free water
- 4.2 SuperScript™ III One-Step RT-PCR System with Platinum™ Taq High Fidelity DNA Polymerase (Invitrogen: Catalog. No. 12574035 – 100 reactions)
- 4.3 SPRI beads (Beckman Coulter: Catalog No. A63880, A63881, A63882, or equivalent)
- 4.4 Molecular biology grade absolute ethanol

5.0 Primers

- 5.1 Universal primers for Influenza A listed in the Table 1 are from Integrated DNA Technologies Inc. (IDT) <http://www.idtdna.com> (or equivalent). Primers must be RNase Free HPLC purified.
 - 5.1.1 Prepare 10 µM stocks of each MBTuni-12, MBTuni12.4, and MBTuni13.
 - 5.1.2 Pool these 10 µM stocks in a 2:3:5 ratio respectively.
- 5.2 CDC provided primers are premixed at 1x strength and dried.
 - 5.2.1 Reconstitute in 1 mL nuclease-free water.

Table 1: MBTuni primer pools			
Oligo	# Bases	Sequence 5'-3'	µM in pool
MBTuni-12	22	ACGCGTGATCAGCAAAAGCAGG	2
MBTuni-12.4	22	ACGCGTGATCAGCGAAAGCAGG	3
MBTuni-13	23	ACGCGTGATCAGTAGAAACAAGG	5



6.0 Samples and Controls

6.1 Samples

- 6.1.1 Specimens collected from individuals with a positive influenza A diagnostic test that have been inactivated and extracted via filter or bead-based RNA extraction platform.
- 6.1.2 Propagated isolate derived from such clinical specimens and similarly inactivated and extracted.

6.2 Controls

Table 2: Positive and Negative Controls

Control	Material	Frequency	Expected Value
Positive	Previously sequenced RNA from propagated isolate of currently circulating influenza A virus, Ct <20 Or Previously sequenced RNA from clinical sample of currently circulating influenza A virus, Ct <20 Or Stock propagated isolates ordered from the International Reagent Resource (IRR) (https://www.internationalreagentresource.org/)	Every run	Amplicons detectable by electrophoresis
Negative	Water	Every run	Amplicons not detectable by electrophoresis

7.0 Safety Precautions

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

8.0 Comments and Questions

- 8.1 Please send comments and questions by email to CDC Influenza Division Technical Support: idseqsupport@cdc.gov



9.0 **Gather Reagents**

- 9.1 Thaw and store at the indicated temperature during the procedure.
- 9.2 Flick/invert the reagent tubes to ensure they are well mixed, and spin down before opening.
- 9.3 **Keep enzymes at in a freezer or on a cold block (approximately -20°C)**
 - 9.3.1 SuperScript III RT Mix
- 9.4 **Keep in a refrigerator, cold block, or ice bucket (approximately 4°C)**
 - 9.4.1 SSIII 2X Reaction Mix
 - 9.4.2 MBTuni Primer Pool
 - 9.4.3 Samples and Controls
- 9.5 **Room temperature (15-25°C).**
 - 9.5.1 Nuclease-free water
 - 9.5.2 SPRI beads (solid-phase reversible immobilization beads)
 - 9.5.2.1 Vortex immediately before use
 - 9.5.3 Freshly prepared 80% ethanol



10.0 MRT-PCR Procedure

- 10.1 Combine the components of Table 3 to prepare a reaction master mix sufficient for all samples and controls.

Table 3: MRT-PCR master mix for influenza A viruses		
Reagent	Volume (µL) Per Reaction	µL Per Master Mix
Nuclease-free Water	8	
2X Reaction Mix	12.5	
SuperScript III RT Mix	0.5	
MBTuni Primer Pool	1	
Subtotal	22	

10.1.1 Aliquot 22 µL of each reaction mix into respective wells of a 96-well PCR plate or into 0.2 mL PCR tubes.

10.1.2 For each sample, positive control, and negative control add 3 µL of RNA or water.

- 10.2 Seal, gently mix, centrifuge, and incubate.

10.2.1 Securely seal to ensure no evaporation occurs.

Table 4: Cycling conditions		
Step	Temperature (°C)	Time (mm:ss)
1	42	50:00
2	50	10:00
3	94	2:00
4	94	0:30
5	43	0:30
6	68	3:50
7	Repeat steps 4-6 for 4 total cycles	
8	94	0:30
9	57	0:30
10	68	3:30**
** Extend this step by 10 seconds per cycle		
11	Repeat steps 8-10 for 30 total cycles	
12	68	10:00
13	4	hold



11.0 Amplicon Quality Control

11.1 QC amplicons via electrophoresis.

11.1.1 PB2: 2.3 kb

11.1.2 PB1: 2.3 kb

11.1.3 PA: 2.2 kb

11.1.4 HA: 1.8 kb

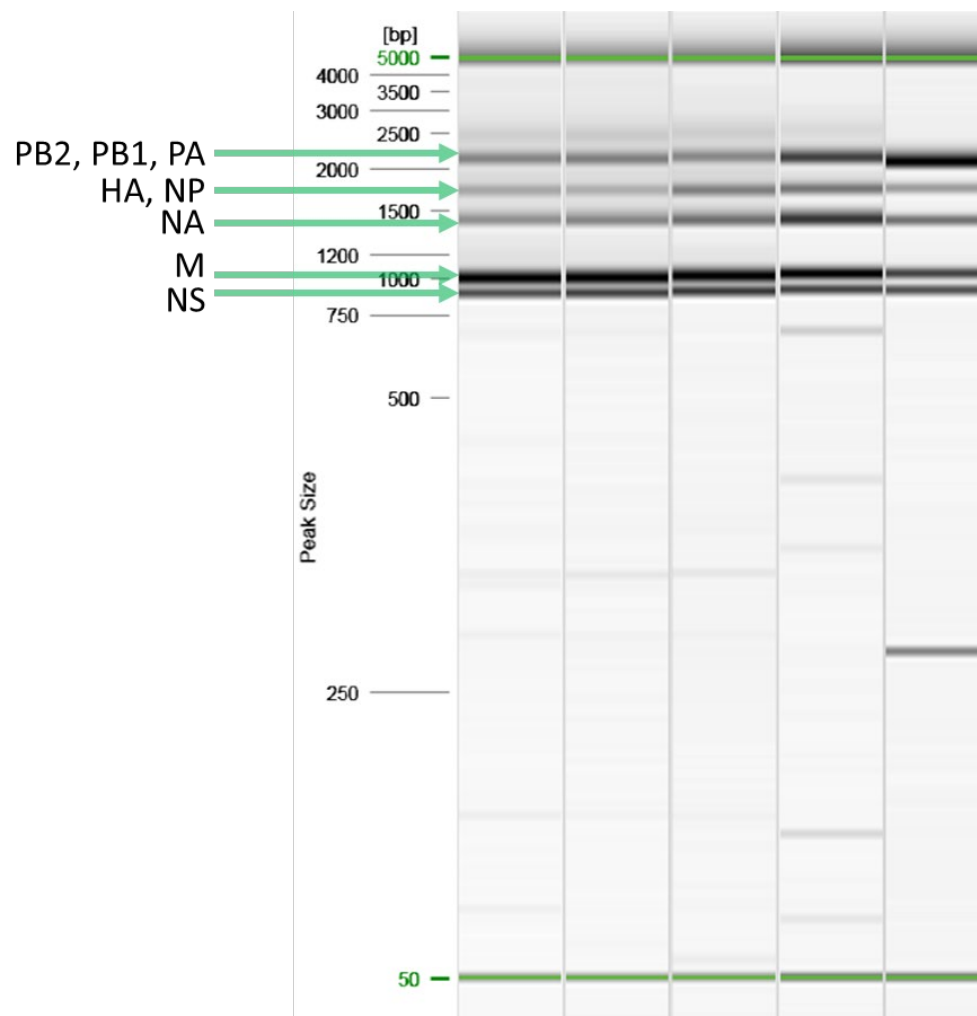
11.1.5 NP: 1.6 kb

11.1.6 NA: 1.4 kb

11.1.7 M: 1.0 kb

11.1.8 NS: 0.9 kb

11.2 **Figure 1:** Example 5-band pattern produced by the MRT-PCR





12.0 SPRI Bead Clean Up with Ethanol Wash and Water Elution

- 12.1 Add 1x (50 μ L) of SPRI beads to each sample, mix gently, and incubate at room temperature for 5 minutes.
- 12.2 Spin down the sample, pellet on a magnet for 2 minutes or until the supernatant is clear.
 - 12.2.1 Remove and discard the supernatant.
- 12.3 With the samples on the magnet and without disturbing the pellet:
 - 12.3.1 Add 200 μ L of 80% ethanol to each sample.
 - 12.3.2 Immediately remove and discard the ethanol.
 - 12.3.2.1 Do not allow the beads to dry to the point of cracking.
 - 12.3.2.2 Proceed immediately to the next step.
- 12.4 With the sample on the magnet and without disturbing the pellet:
 - 12.4.1 Add 200 μ L of 80% ethanol to each sample.
 - 12.4.2 Immediately remove and discard the ethanol.
 - 12.4.2.1 Do not allow the beads to dry to the point of cracking.
 - 12.4.2.2 Proceed immediately to the next step.
- 12.5 Spin down the samples, pellet on a magnet for 10 seconds or until the beads collect to one side.
 - 12.5.1 Remove and discard any residual ethanol.
 - 12.5.1.1 Do not allow the beads to dry to the point of cracking.
 - 12.5.1.2 Proceed immediately to the next step.
- 12.6 Remove from the magnet, add 15 μ L of water to each sample, gently resuspend, and incubate at room temperature for 10 minutes.
- 12.7 Spin down the sample, pellet on a magnet for 2 minutes or until the supernatant is clear.
 - 12.7.1 Remove and retain the supernatant (cleaned amplicons) in a new plate.

13.0 Proceed to Sequencing

- 13.1 The amplicons produced here are suitable for nanopore sequencing as described in LP-512 – Native Library Preparation and Nanopore Sequencing of Influenza A Virus and SARS-CoV-2 S-gene Amplicons.
- 13.2 The user may decide to sequence the amplicons produced here via other methods of library preparation and or other sequencing platforms that are suitable for 0.9-2.3 kb amplicons; however, it is the responsibility of that user to validate the chosen sequencing method. It is not recommended to use sequencing data that is of partial or low coverage or low quality.



14.0 **Related Procedures**

- 14.1 LP-512 – Native Library Preparation and Nanopore Sequencing of Influenza A Virus and SARS-CoV-2 S-gene Amplicons

15.0 **References**

- 15.1 Product information sheet: SuperScript™ III One-Step RT-PCR System with Platinum™ Taq DNA Polymerase (Invitrogen)
- 15.2 Biosafety in Microbiological and Biomedical Laboratories (BMBL), current edition
<https://www.cdc.gov/labs/BMBL.html>