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Optimized RT-PCR Protocols for Whole Genome Amplification of Influenza A Virus for NGS

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NGS Optimization

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We use this protocol and it's working

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Abstract

This protocol describes optimized protocols for simultaneous amplification of all 8 influenza A segments using the Opti primer set with the SuperScript IV one-step RT-PCR kit or LunaScript Multiplex One-Step RT-PCR Kit. Purified amplicons can be used for NGS library preparation for influenza whole-genome sequencing.

Troubleshooting

Materials & Consumables

1 **Materials**

5 µl of Influenza A RNA

2 **Consumables**

- SuperScript IV One-Step RT-PCR System (Invitrogen, cat. number 12594100 or 12594025) or LunaScript Multiplex One-Step RT-PCR Kit (NEB, cat. number E1555S or E1555L)
- Opti primers:

	COMPONENT	SEQUENCE (5' → 3')
	Opti F1	GTT ACG CGC CAG CAA AAG CAG G
	Opti F2	GTT ACG CGC CAG CGA AAG CAG G
	Opti R	GTT ACG CGC CAG TAG AAA CAA GG

OPTI Primer Mix

3 First, create a master [M] 100 micromolar (µM) stock solution for each primer and then dilute it to a [M] 20 micromolar (µM) working solution mix. This reduces the number of freeze/thaw cycles that the primer stock goes through and reduces the chances of contamination.

4 **Preparation of Primer Stock Solutions (100 µM)** 10s

Note

The microliters of water required to create a [M] 100 micromolar (µM) solution is 10x the nanomoles of lyophilized primer (i.e., if 26.5 nmol is noted on side of tube, a 100 µM primer stock solution is created by adding 265 µL of water or TE buffer to stock tube). The original primer tubes are used for a 100 µM stock.

- Find the oligo yield information in nmol on your tube label

- Multiply this number by 10. The resulting product is the amount of water or TE buffer needed (in µL)
- Reconstitute lyophilized primers in required volume of molecular grade water (i.e., RNase/DNase free) or TE buffer
- Once water has been added, vortex for at least 00:00:10 to ensure the lyophilized primers are fully dissolved and store at -20 °C to use as needed

5 **Preparation of Primer Working Solutions (20 µM)** 20s

In a clean template-free pre-PCR hood, prepare working solution of the OPTI primer mix:

- Allow stock solution to thaw completely at Room temperature
- Vortex the stock solution for at least 00:00:10 to ensure homogenization before adding it to the working solution
- Prepare a [M] 20 micromolar (µM) working solution by combining stock solutions (100 µM) of Opti F1/F2/R primers in 0.35:0.65:1 ratio:

	COMPONENT	CONCENTRATION	VOLUME
	Nuclease-free water	-	80 µl
	Opti F1	100 µM	3.5 µl
	Opti F2	100 µM	6.5 µl
	Opti R	100 µM	10 µl
	TOTAL		100 µl

Note

100 µl of Opti primer mix sufficient to perform 40 reactions at a 50-µL RT-PCR reaction volume

- Once the concentrated stock is added, vortex the new working solution for at least 00:00:10
- To maintain optimal use of the primers, limit freeze/thaw cycles, as this can lead to degradation over time. To avoid repeated thawing and freezing, prepare small aliquots of working solutions and store at -20 °C. Thawed working primer solutions can be kept at 4 °C for weeks or months to use as needed.

6 Select one of the two one-step RT-PCR kit protocols below:
- SuperScript IV One-Step RT-PCR System (Invitrogen)
- LunaScript Multiplex One-Step RT-PCR Kit (NEB)

STEP CASE

SuperScript IV One-Step RT-PCR System (Invitrogen) 4 steps

7 Preheat the thermocycler to 55 °C, with the heated lid set to 105 °C

8 Keep all components, reaction mixes, and samples On ice

9 Prepare a 25 µL Master Mix (Volume 1) per reaction if visualization on TapeStation or no visualization is intended, and a 50 µL Master Mix (Volume 2) if gel visualization after PCR is planned for amplification product verification. Mix the following components:

	COMPONENT	VOLUME 1	VOLUME 2
	Nuclease-free water	8.5 µl	17 µl
	2X Platinum™ SuperFi™ RT-PCR Master Mix	12.5 µl	25 µl
	Opti primer mix (20 µM working solution)	1.25 µl	2.5 µl
	SuperScript™ IV RT Mix	0.25 µl	0.5 µl
	RNA template	2.5 µl	5 µl
	TOTAL	**25 µl**	**50 µl**

Mix gently, briefly centrifuge, and ensure all the components are at the bottom of the amplification tube

10 Place the reaction tubes in the pre-heated thermocycler and select the following RT-PCR cycling conditions:

	STEP	TEMPERATURE	TIME	CYCLE
	Reverse transcription	55˚C	2 min	1
		42˚C	90 min	

	STEP	TEMPERATURE	TIME	CYCLE
	RT inactivation/initial denaturation	98˚C	2 min	1
	Amplification 1	98˚C	10 sec	5
		45˚C	30 sec	
		72˚C	3.5 min	
	Amplification2	98˚C	10 sec	30
		67˚C	30 sec	
		72˚C	3.5 min	
	Final extension	72˚C	10 min	1
	Hold	4˚C	∞	-

Note

Safe Stop Point: If necessary, the protocol can be paused and samples can be kept at 4 °C overnight or at -20 °C for long-term storage, before proceeding to downstream applications.

For downstream NGS library construction, a cleanup step with **Select-a-Size DNA Clean & Concentrator** (Zymo Research, cat. number D4080) is recommended, following the manufacturer's protocol to retain DNA fragments ≥ 300 bp.