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Influenza A, B amplicon-based sequencing protocol using Illumina DNA preplibrary preparation V.1

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We use this protocol and it's working

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Abstract

Influenza A and B viruses are major contributors to seasonal respiratory infections and global health burdens. Accurate and timely genomic surveillance of these viruses is critical for monitoring viral evolution, informing vaccine strain selection, and detecting antiviral resistance. This protocol outlines a streamlined workflow for whole-genome sequencing (WGS) of Influenza A and B viruses directly from clinical samples or viral isolates. The process includes RNA extraction, reverse transcription using universal influenza primers, multiplex PCR amplification of all gene segments, library preparation using a tagmentation-based approach, and sequencing on an Illumina platform. Bioinformatic analysis involves quality control, genome assembly, and variant calling using reference-guided alignment. The protocol enables high-throughput, cost-effective sequencing with high genome coverage and accuracy. It is suitable for public health laboratories and research institutions engaged in influenza surveillance and evolutionary studies.

Materials

Reagents:

- Illumina DNA/RNA UD Indexes Set B, Tagmentation (96 Indexes, 96 Samples) **Illumina, Inc. Catalog #**20091656
- Illumina DNA/RNA UD Indexes Set B, Tagmentation (96 Indexes, 96 Samples) **Illumina, Inc. Catalog #**20091656
- Illumina DNA/RNA UD Indexes Set C, Tagmentation (96 Indexes, 96 Samples) **Illumina, Inc. Catalog #**20091658
- Illumina DNA Prep, (M) Tagmentation (96 Samples, IPB) **Illumina, Inc. Catalog #**20060059
- Illumina DNA/RNA UD Indexes Set D, Tagmentation (96 Indexes, 96 Samples) **Illumina, Inc. Catalog #**20091660
- Illumina DNA/RNA UD Indexes Set A, Tagmentation (96 Indexes, 96 Samples) **Illumina, Inc. Catalog #**20091654
- Ethanol absolute ≥99.8% **VWR International (Avantor) Catalog #**20821.365
- Qubit™ dsDNA Quantification HS Assay Kits **Thermo Scientific Catalog #**Q32854
- Illumina DNA Prep, (M) Tagmentation (96 Samples, IPB) **Illumina, Inc. Catalog #**20060059
- Nuclease-Free Water **Thermo Fisher Scientific Catalog #**AM9932

- Pipette Tips, sterile, filtered (assorted volumes)
- Conical Tubes, 10ml and/or 15ml (FisherSci cat# 14-959-53A or equivalent)
- Solution basins, sterile (FisherSci cat# 13-681-504 or equivalent)
- 96-well PCR Plates, semi-skirted, flat deck (FisherSci cat# AB-1400L or equivalent)
- Microcentrifuge tubes, 1.5 ml, sterile (Thermofisher cat# AM12400 or equivalent)
- Plate Seals (FisherSci cat# AB-0558 or equivalent)

Qubit Flex Fluorometer (Cat. No. Q33326)
• Qubit Flex Assay Tube Strips, 125 strips (Cat. No. Q33252)
• Qubit Flex System Verification Assay Kit (Cat. No. Q33254)
• Qubit Flex Reservoirs, 100 reservoirs (Cat. No. Q33253)

Consumables:

1. Qubit Flex 4.0
2. Thermocycler with heated lid
3. Microplate centrifuge
4. Vortex

5. Magnetic Stand-96 (Thermofisher cat# AM10027) (If possible, have two; one for pre-PCR and one for post-PCR)
6. Micropipettes (Single and Multichannel)
7. Ice bucket
8. Microcentrifuge

Protocol materials

- Illumina DNA/RNA UD Indexes Set B, Tagmentation (96 Indexes, 96 Samples) **Illumina, Inc. Catalog #**20091656
- Illumina DNA/RNA UD Indexes Set C, Tagmentation (96 Indexes, 96 Samples) **Illumina, Inc. Catalog #**20091658
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- Illumina DNA/RNA UD Indexes Set A, Tagmentation (96 Indexes, 96 Samples) **Illumina, Inc. Catalog #**20091654
- Nuclease-Free Water **Thermo Fisher Scientific Catalog #**AM9932
- TURBO DNase 2 U/uL **Fisher Scientific Catalog #**AM2239
- Ethanol absolute, KOPTEC, meets analytical specification of BP, Ph. Eur., USP (200 Proof) **Avantor Sciences Catalog #**89125-176
- AMPure XP Beads (AXP) **Beckman Coulter Catalog #**AXP
- Illumina® DNA/RNA UD Indexes Set A, B, C, D Tagmentation (96 Indexes, 96 Samples) **Illumina, Inc. Catalog #**20091654, 20091656, 20091658, 20

Safety warnings

Always use IC during the PCR and alwaxs check the RNA integrity!

RNA extraction

1 RNA extraction
Samples were extracted using QIAamp Viral RNA Mini kit (Catalog # 52904). Real time RT-PCR (CDC 2009) was performed on all samples to determine viral load with Cycle threshold (Ct) value. Samples with a Ct value <32 are recommended for optimal results. RNA integrity affect the quality and the yield of Library!

DNAse treatment

2 DNAse digestion by using Turbo DNAse
TURBO DNase 2 U/uL **Fisher Scientific Catalog #**AM2239
Create a master mix of Turbe DNAse:

Add 1 µL TURBO DNase (2 U) (for up to 10 µg RNA in a 50 µL reaction), 5µL TURBO DNase Buffer, and 14µL of H2O per sample.
30:00 min 37 °C

cDNA synthesis and amplification

3 Reagents:
Invitrogen™ SuperScript™ III One-Step RT-PCR System with Platinum™TaqHigh Fidelity DNA Polymerase (cat. no. 12574035)

Oligos for amplicon generation (7,8):

	A	B
	Primer	Sequence
	Uni12/Inf-1	GGGGGGAGCAAAAGCAGG
	Uni12/Inf-3	GGGGGGAGCGAAAGCAGG
	Uni13/Inf-1	CGGGTTATTAGTAGAAACAAGG

	A	B
	B-PBs-UniF	GGGGGGAGCAGAAGCGGAGC
	B-PBs-UniR	CCGGGTTATTAGTAGAAACACGAGC
	B-PA-UniF	GGGGGGAGCAGAAGCGGTGC
	B-PA-UniR	CCGGGTTATTAGTAGAAACACGTGC
	B-HANA-UniF	GGGGGGAGCAGAAGCAGAGC
	B-HANA-UniR	CCGGGTTATTAGTAGTAACAAGAGC
	B-NP-UniF	GGGGGGAGCAGAAGCACAGC
	B-NP-UniR	CCGGGTTATTAGTAGAAACAACAGC
	B-M-Uni3F	GGGGGGAGCAGAAGCACGCACTT
	B-Mg-Uni3F	GGGGGGAGCAGAAGCAGGCACTT
	B-M-Uni3R	CCGGGTTATTAGTAGAAACAACGCACTT
	B-NS-Uni3F	GGGGGGAGCAGAAGCAGAGGATT
	B-NS-Uni3R	CCGGGTTATTAGTAGTAACAAGAGGATT

PCR mix:

	A	B
	Components	**Volume (µl) for 1 x rxn**
	SuperScript III RT/ Platinum Taq High Fidelity Enzyme Mix	0.5
	2X Reaction Mix (a buffer containing 0.4 mM of each dNTP	12,5

	A	B
	RNAse/DNAse free water	5.5
	primer pool (10 µM)	2,5
	Total volume	20
	Template RNA (1 pg to 1 µg)*	**5**
	Final volume*	**25.0**

* scale up if needed

PCR conditions:

	A	B	C	D
	Step	**Temperature**	**Duration**	**Number of cycle**
	Reverse transcription	42°C	60 mins	1x
	Activation/initial denaturation	94°C	2 mins	1x
	Amplification	94°C	15 sec	7x
		44°C	30 sec	
		68°C	5 min	
		94 °C	20 sec	35x
		58 °C	1 min	
		68 °C	5 min	
	Final extension	68 °C	10 min	1x
	HOLD	12°C	infinite	infinite

Note:

- Keep all components, reaction mixes, and samples on ice. After preparation of the samples, transfer them to the preheated thermal cycler and immediately start the RT–PCR program.
- If the ct vaue is higher than the expected <32, scale up reactions

cDNA synthesis and amplification

4 **Clean up with Ampure bead**

AMPure XP Beads (AXP) **Beckman Coulter Catalog #**AXP

Ethanol absolute, KOPTEC, meets analytical specification of BP, Ph. Eur., USP (200 Proof) **Avantor Sciences Catalog #**89125-176

Nuclease-Free Water **Thermo Fisher Scientific Catalog #**AM9932

1. Take the beads to room temperature before use!
2. Shake the AMPure XP bottle to resuspend any magnetic particles that may have settled. Then add 1,8x AMPure XP reagent.
3. Mix reagent and sample thoroughly by pipette mixing 10 times.
Let the mixed samples incubate for 5 minutes at room temperature for maximum recovery.
4. Place the reaction plate onto the **SPRIPlate 96R Ring Super Magnet Plate** for 2 minutes to separate beads from the solution. Wait for the solution to clear before proceeding to the next step.

5. Perform this step with the reaction plate situated on the SPRIPlate 96R Ring Super Magnet Plate. Do not
disturb the separated magnetic beads. Also, be sure to remove all of the ethanol from the bottom of the well. Dispense 200 µL of 80% ethanol to each well of the reaction plate and incubate for 30 seconds at room temperature. Aspirate out the ethanol and discard.
6. Repeat for a total of two washes.
7. Dry the beads and do not over dry the bead ring (bead ring appears cracked if over dried) as this
will significantly decrease elution efficiency.
8. Remove the reaction plate from the magnet plate, and then add 52 µL of elution buffer (RNAse/DNAse-free water) to each well of the reaction plate and pipette mix 10 times. Incubate for 2 minutes.
9.Place the reaction plate onto the SPRIPlate 96R Ring Super Magnet Plate for 1 minute to separate beads from the solution.
10. Transfer 50 ul of the eluate to a new plate.

QC using Qubit

5 Quantify samples using the Qubit dsDNA High Sensitivity kit. See SOP titled“DNA Quantification using the Qubit Fluorometer” for more detailed information on performing DNA quantification.

Library preparation with Illumina DNAprep

6 **Tagmenation**

Consumables:

BLT (Bead-Linked Transposomes)

TB1 (Tagmentation Buffer 1)

- Nuclease-free water
- 8-tube strip
- 96-well PCR plate
- Microseal 'B' adhesive seal
- 20 µl Pipette tips
- 200 µl Pipette tips

Mix :

BLT (11 µl)

TB1 (11 µl)

1.Use a new plate and add 20 ul mix to 30 ul previously eluated sample.

2. Gently pipette up and down 10 times

3. Seal the plate

4. Centrifuge down the samples (gently)

5. Incubate the samples as a follows:

37 °C 15:00 min 10 °C

Clean up with TWB

7 1. Remove the plate seal.
2. Add 10ul of TSB to each sample (Use a multi-channel pipette) and gently pipette up and down 10 times to mix and fully resuspend the beads in the 50 µl reaction.
3. Apply an adhesive PCR plate seal to the plate.

4. Place the plate into the thermocyler and incubate at for 37 °C followed by a 10 °C hold
5. While samples are incubating, thaw EPM on ice.
6. Remove the plate from the thermocycler, quick spin the plate and remove the seal.
7. Place the plate on a magnet and incubate it until solution is clear.
8. Using a multichannel pipette, remove the supernatant and discard.
9. Remove the plate from the magnet and add of TWB directly to the pellet.
10. Gently pipette to mix until beads are fully resuspended, try to avoid creation of foam from TWB.
11. Place the plate on the magnet for or until solution is clear.
12. Remove the supernatant and discard.
13. Remove the plate from the magnet and add of TWB directly to the pellet.
14. Gently pipette to mix until beads are fully resuspended.
15. Place the plate on the magnet for 00:03:00 or until solution is clear.
16. Remove the supernatant and discard.
17. Remove the plate from the magnet and add 100 µL of TWB directly to the pellet. Gently pipette to mix until beads are fully resuspended.

18. Place the plate with TWB on the magnet and avoid overdrying and incubate until ready to proceed with adding the PCR master mix in the Amplify Tagmented DNA step.

8 **Amplification and indexing**

Vortex the EPM to mix, then briefly centrifuge.
Add:
22 ul EPM and
22 ul Water

19. Remove and discard TWB. Avoid overdrying!
20. Add 40 ul EPM mix to the beads.
21. Add 10 ul Illumina UD index to the mix

Illumina® DNA/RNA UD Indexes Set A, B, C, D Tagmentation (96 Indexes, 96 Samples) **Illumina, Inc. Catalog #**20091654, 20091656, 20091658, 20

Incubate as a follows:

PCR conditions:

	A	B	C	D
	Step	Temperature	Duration	Number of cycle

	A	B	C	D
	Activation/initial denaturation	68°C	3 mins	1x
		98°C	3 mins	
	Amplification			
		98°C	45 sec	12x
		62°C	30 sec	
		68 °C	2 min	
	Final extension	68 °C	2 min	1x
	HOLD	12°C	infinite	infinite

9 Clean up libraries

Centrifuge at 280 × g for 1 minute to collect contents at the bottom of the well.

2. Place the plate on the magnetic stand and wait until the liquid is clear (~5 minutes).
3. Transfer 45 µl supernatant from each well of the a PCR plate.
4. Resuspend IPB
5. Add 40 µl nuclease-free water to each well-containing supernatant.
6. Add 45 µl IPB to each well-containing supernatant.
7. Pipette each well 10 times to mix.
8. Seal the plate and incubate at room temperature for 5 minutes.
9. Place on the magnetic stand and wait until the liquid is clear (~5 minutes).
10. Add 15 µl to each well of a new MIDI plate.
11. Transfer 125 µl supernatant from each well the new MIDI plate containing 15 µl IPB.
12. Incubate the sealed MIDI plate at room temperature for 5 minutes.
13. Place on the magnetic stand and waituntil the liquid is clear (~5 minutes).
14. Without disturbing the beads, remove and discard supernatant.
15. Wash beads as follows.
16. With the plate on the magnetic stand, add 200 µl fresh 80% EtOH without mixing.
17. Incubate for 30 seconds.
18. Without disturbing the beads, remove and discard supernatant.
19. Wash beads a second time.

20. Use a 20 µl pipette to remove and discard residual EtOH.
21. Air-dry on the magnetic stand for 5 minutes.
22. Remove from the magnetic stand.
23. Add 32 µl RSB to the beads.
24. Pipette to resuspend.
25. Incubate at room temperature for 2 minutes.
26. Place the plate on the magnetic stand and wait until the liquid is clear (~2 minutes).
27. Transfer 30 µl supernatant to a new 96-well PCR plate.

SAFE STOPPING POINT

If you are stopping, seal the plate with Microseal 'B' adhesive seal or Microseal 'F' foil seal, and store at -25°C to -15°C for up to 30 days.

9.1

SAFE STOPPING POINT

If you are stopping,
seal the plate with Microseal 'B' adhesive seal or Microseal 'F' foil seal, and store at -25°C to -15°C for up to 30 days.

10 **Pool Libraries**

Combine 5-10 ul of libraries.

Dilute pooled libraries according to the Illumina Denature and dilute protocols.

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