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Seasonal and zoonotic influenza WGS using ONT rapid barcoding technology V.1

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We use this protocol and it's working

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Abstract

This protocol is suitable for seasonal and zoonotic rapid whole genome sequencing using universal primers for influenza A and Oxford Nanopore Technologies (ONT) rapid chemistry (SQK-RBK114.24 or SQK-RBK114.96). Using ONT and tierce bioinformatic tools, it enables sub-typing and clade resolution sequencing result in one working-day.

Nucleic acid extraction

1h

1 RNA extraction is performed using either Maxwell® CSC Pathogen Total Nucleic Acid Kit **AS1860** or EMAG® Nucleic Acid Extraction System Generic program, allowing the management of up to 16 or 24 samples simustaneously depending on the extraction system.

1h

Universal Influenza A Primers

2 Univ primers are used for the 8 segments amplification. Primers sequences are listed here :

	Primers	5'-3' Sequences
	Uni12 Forward (Fw)	GGG-GGG-AGC-AAA-AGC-AGG
	Uni12b Forward (Fw) (Uni12_inf3)	GGG-GGG-AGC-GAA-AGC-AGG
	Uni13 Reverse (Rv)	CGG-GTT-ATT-AGT-AGA-AAC-AAG-G

Table 1 : Universal Influenza A Primers

Pre-PCR and amplification

4h

3 Pre-PCR is prepared as follows :

- ezDNase **11766051** (Thermofisher Scientific) is used for nucleic-acid extracts DNase treatment

	Reagent	Volume / Sample (µL)
	nuclease-free water	0.625
	ezDNase buffer (10X)	1.25
	ezDNase enzyme	0.625
	nucleic acid extract	10
	Total	12.5

Table 2 : Nucleic-acid extract DNase treatment

DNase treatment is performed by incubating the prepared mix at 37 °C for 00:10:00

3.1 SuperScript III One Step RT-PCR System **12574035** (Thermofisher Scientific) combined with RNasin **N2511** (Promega) is then used as follows :

	Reagent	Volume / Sample (µL)
	2X Reaction Mix	12.5
	ezDNase - nucleic acid extract mix	5
	nuclease-free water	4.75
	Primer Uni12 forward (10µM)	0.5
	Primer Uni12b forward (10µM)	0.5
	Primer Uni13 reverse (10µM)	1
	RNasin	0.25
	SuperScript III RT/ Platinum Taq High Fidelity Enzyme Mix	0.5
	Total	20

Table 3 : Mix preparation for one-step RT-PCR

One-step RT-PCR is performed on a thermocycler as follows 03:30:00 :

	Temp (C°)	Time	Cycle(s)
	45	01:00:00	1
	94	00:02:00	
	94	00:00:30	5
	45	00:00:30	

	Temp (C°)	Time	Cycle(s)
	68	00:03:00	
	94	00:0030	31
	57	00:00:30	
	68	00:03:00	

Table 4 : Thermocycler one-step RT-PCR program

Library preparation

4 The library construction is performed according to manufacturer's instruction for the Rapid Barcoding Kit (SQK-RBK114.24/96).
Before starting this step it's possible to perform a flow cell check on the ONT device to gain some time on the whole experiment.

Briefly, each sample is quantified using QuBIt fluorometer and normalised to obtain 50 ng of amplified DNA in a 10 µL volume.
1.5 µL of rapid barcode are added to the reaction volume and the mix is then incubated as follows :

30 °C 00:02:00
80 °C 00:02:00

4.1 Once the incubation time is over, each sample is pooled in a single 1.5mL LoBind Eppendorf tube and a 1:1 AMPure XP beads purification is performed. Two 80% ethanol washes are performed on a magnetic rack. Elution is done in 11 µL of Elution Buffer (EB)

4.2 Rapid Adapter mix is performed as follows :

	Reagent	Volume (µL)
	Adapter Buffer (ADB)	3.5

	Reagent	Volume (µL)
	Rapid Adapter (RA)	1.5
	Total	5

Table 5 : Rapid Adapter Mix (unused mix can be stored at -20°C for further use)

1 µL of Rapid Adapter mix is added to the 11 µL of previously barcoded and purified samples.
The resulting mix is incubated 00:05:00 at room temperature and then stocked at 4°C prior to loading on the flow cell

Sequencing and analysis

1h 30m

5 Sequencing is performed on any ONT sequencing device. Preferentially use one with computing capacity allowing for real-time High Accuracy basecalling mode (HAC). 1h

R10.4.1 flow cell is loaded according to manufacturer's instruction and sequencing run is launched for at least 01:00:00 for small batches of samples (<8). The time required for successful sequencing is depending on samples multiplexing but also individual samples overall quality and viral load thus should be tested to ensure good results. Once the short run is over, it can be re-launched for a longer time to use as supplementary data if needed.

5.1 Analysis can be performed immediately using Epi2Me wf-flu (https://github.com/epi2me-labs/wf-flu). 30m
The RBK kit usage must be specified while setting-up the analysis run.

The wf-flu is not suited for zoonotic influenza type so the samples will always be Undetermined in the subtyping results of the report. If there is a suspicion for zoonotic influenza, an alternative solution can be used as described after.
Using intermediate files generated in the analysis directory, it's still possible to obtain the consensus for the samples of interest (wf-flu_ID\output\SampleID\consensus\draft.consensus.fasta).
The consensus can then be uploaded on Nextclade with the automatically suggested reference dataset, allowing for clade determination based on the HA sequence