DEPARTMENT OF HEALTH & HUMAN SERVICES Public Health Service
Centers for Disease Control and Prevention (CDC)
Atlanta, GA 30329

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

Virology, Surveillance and Diagnosis Branch, Genomics and Diagnostics Team (GDT)

1.0 Purpose

1.1 This procedure describes the library preparation and nanopore sequencing of amplicons from:

1.1.1 FluSeq: LP-497 – MRT-PCR of Influenza A Viruses for Sequencing

1.1.2 SpikeSeq: LP-471 – RT-PCR the SARS-CoV-2 S-gene for Sequencing

2.0 Definitions

2.1 Library Preparation: The modification of nucleic acids into a suitable state as to be loaded onto a sequencing device.

2.2 FluSeq: Amplification and nanopore sequencing of the influenza A viral genome

2.3 SpikeSeq: Amplification and nanopore sequencing of the SARS-CoV-2 S-gene

2.4 Room temperature: 15-25°C

3.0 Equipment (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. AXYCMFLAT)

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 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)

3.7 Pipettes (10 µL, 20 µL, 200 µL, and 1000 µL)

3.7.1 Multichannel pipettes also recommended (10 µL, 20 µL, and 200 µL)

3.7.2 Corresponding aerosol barrier pipette tips

3.8 Disposable reagent reservoir, sterile (Axygen: Catalog No. RES-V-25-S)

3.9 96-well format PCR Thermocycler System (BioRad T100)

3.10 Qubit fluorometer and tubes for DNA quantification (Thermo Fischer: Catalog No. Q33238)

3.11 Mk1C or GridION nanopore sequencing device (Oxford Nanopore Technologies)

4.0 Reagents

4.1 Nuclease-free water

4.2 SPRI beads (Beckman Coulter: Catalog No. A63880, A63881, A63882, or equivalent)

4.3 Molecular biology grade absolute ethanol

4.4 Blunt/TA Ligase Master Mix (NEB: Catalog No. M0367)

4.5 NEBNext Ultra II End repair/dA-tailing module (NEB: Catalog No. E7546)

4.6 NEBNext Quick Ligation Module (NEB: Catalog No. E6056)

4.7 Native barcoding kit 96 V14 (Oxford Nanopore Technologies: Catalog No. SQK-NBD114.96)

4.8 Nanopore flow cells: disposable flongle flow cells and or standard MinION flow cells

4.8.1 Disposable flongle flow cells (Oxford Nanopore Technologies: Catalog No. FLGIntSP and or FLO-FLG114)

4.8.1.1 Flongle Sequencing Expansion kit (EXP-FSE002, ships with flongle flow cells)

4.8.1.2 Flongle Adapter (Included with FLGIntSP)

4.8.2 Standard MinION flow cells (Oxford Nanopore Technologies: Catalog No. FLO-MIN114)

4.8.2.1 Must be returned to Oxford Nanopore Technologies

4.9 Qubit dsDNA HS assay kit (Thermo Fisher Scientific: Catalog No. Q32851 – 100 assays or Q32854 – 500 assays)

5.0 Samples and Controls

5.1 All sequencing runs should include positive and negative controls.

5.2 Samples and controls should cleaned and quality control tested amplicons carried over from:

5.2.1 FluSeq: LP-497 – MRT-PCR of Influenza A Viruses for Sequencing

5.2.1.1 Optional: Normalize cleaned amplicons to 20 ng/µL each.

5.2.1.2 FLO-FLG114: up to 24 samples may be pooled per library

5.2.1.3 FLO-MIN114: up to 48 samples may be pooled per library

5.2.2 SpikeSeq: LP-471 – RT-PCR the SARS-CoV-2 S-gene for Sequencing

5.2.2.1 Optional: Normalize cleaned amplicons to 30 ng/µL each.

5.2.2.2 FLO-FLG114: up to 48 samples may be pooled per library

5.2.2.3 FLO-MIN114: up to 96 samples may be pooled per library

6.0 Safety Precautions

6.1 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).

7.0 Comments and Questions

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

8.0 Gather Reagents

8.1 Thaw and store at the indicated temperature during the procedure.

8.2 Flick/invert the reagent tubes to ensure they are well mixed, and spin down before opening.

8.3 **Keep enzymes in a freezer or on a cold block (approximately -20°C)**

- 8.3.1 Ultra II End Prep Enzyme Mix (Do Not Vortex)
- 8.3.2 Blunt TA MM (blunt/thymine adenine ligase master mix)
- 8.3.3 Quick T4 DNA Ligase

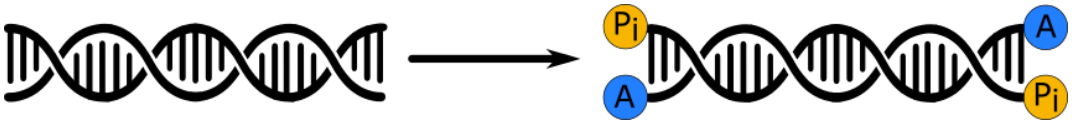
8.4 **Keep in a refrigerator, cold block, or ice bucket (approximately 4°C)**

- 8.4.1 Cleaned Amplicons from LP-497 or LP-471
- 8.4.2 DCS (DNA Control Sample)
  - 8.4.2.1 Either a previously EB diluted tube or a new tube
- 8.4.3 Ultra II End Prep Buffer (May contain precipitate. Bring to room temperature and vortex to dissolve. Then return to ~4°C for use during the procedure)
- 8.4.4 96 well plate of native barcodes
- 8.4.5 5x Quick Ligation Buffer
- 8.4.6 NA (Native Adapter)
- 8.4.7 EDTA
- 8.4.8 SFB (Short Fragment Buffer)
- 8.4.9 EB (Elution Buffer)
- 8.4.10 SB (Sequencing Buffer, glass vial for flongle)
- 8.4.11 LIB (Library Beads, glass vial for flongle)
- 8.4.12 FCF (Flow Cell Flush, glass vial for flongle)
- 8.4.13 FCT (Flow Cell Tether)
- 8.4.14 Qubit buffer and standards

8.5 **Room temperature (15-25°C).**

- 8.5.1 Nuclease-free water
- 8.5.2 SPRI beads (solid-phase reversible immobilization beads)
  - 8.5.2.1 Vortex immediately before use
- 8.5.3 Freshly prepared 80% ethanol
- 8.5.4 Qubit dye

9.0 End-Repair



9.1 Add 105 µL of Elution Buffer (EB) to a new tube of DNA Control Sample (DCS)

9.1.1 Mark the tube to note that it has been diluted

9.1.2 Save the remaining diluted DCS for future library preparations

9.2 Prepare a master mix:

Table 1: End-Repair master mix		
Reagent	**Volume (µL) Per Reaction**	**µL Per Master Mix**
Diluted DCS	1	
Ultra II End-Prep Buffer	1.75	
Ultra II End-Prep Enzyme	0.75	
Total	**3.5**	

9.3 For each sample, transfer 3.5 µL of the end-prep master mix to the wells of a new plate.

9.4 For each sample add 11.5 µL of cleaned amplicons to the end-prep master mix containing wells.

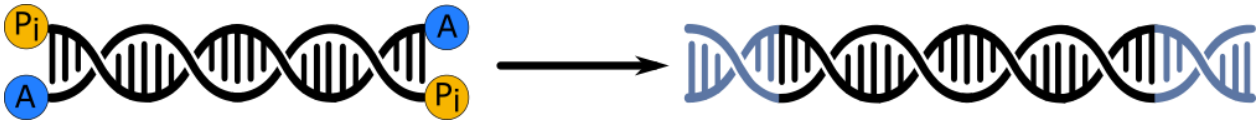
9.4.1 Seal and retain the remaining the clean amplicons.

9.5 Seal, mix, centrifuge, and incubate the end-prep plate.

9.5.1 Securely seal to ensure no evaporation occurs.

Table 2: End-Prep incubation conditions: 15 µL	
Temperature (°C)	**Time (mm:ss)**
20	5:00
65	5:00
4	hold

10.0 Native Barcoding



10.1 Prepare a master mix of diluted Blunt TA Master Mix for each sample:

Table 3: Diluted Blunt TA Master Mix		
Reagent	**Volume (µL) Per Reaction**	**µL Per Master Mix**
Nuclease-Free Water	3	
Blunt TA Master Mix	5	
Total	**8**	

10.2 Add to each well of a new plate:

Table 4: Native barcoding reaction	
Reagent	**Volume (µL)**
Diluted Blunt TA Master Mix	8
Unique Native Barcodes	1.25
End prepped samples	0.75
Total	**10**

10.2.1 Seal and retain the remaining material in end-prep plate.

10.3 Seal, mix, centrifuge, and incubate the barcoding plate at room temperature for 20 minutes.

10.4 Add 1 µL of EDTA and mix to stop the reaction.

10.5 Pool the samples and note the volume.

11.0 SPRI Bead Clean Up with Ethanol Wash and Water Elution

11.1 Add 0.4x of SPRI beads, mix gently, and incubate at room temperature for 5 minutes.

$$volume\ of\ pool\ (\mu L) \times 0.4 = volume\ of\ beads\ (\mu L)$$

11.2 Spin down the sample, pellet on a magnet for 2 minutes or until the supernatant is clear.

11.2.1 Remove and discard the supernatant.

11.3 With the sample on the magnet and without disturbing the pellet:

11.3.1 Add 700 µL of 80% ethanol.

11.3.2 Immediately remove and discard the ethanol.

11.3.2.1 Do not allow the beads to dry to the point of cracking.

11.3.2.2 Proceed immediately to the next step.

11.4 With the sample on the magnet and without disturbing the pellet:

11.4.1 Add 700 µL of 80% ethanol.

11.4.2 Immediately remove and discard the ethanol.

11.4.2.1 Do not allow the beads to dry to the point of cracking.

11.4.2.2 Proceed immediately to the next step.

11.5 Spin down the sample, pellet on a magnet for 10 seconds or until the beads collect to one side.

11.5.1 Remove and discard any residual ethanol.

11.5.1.1 Do not allow the beads to dry to the point of cracking.

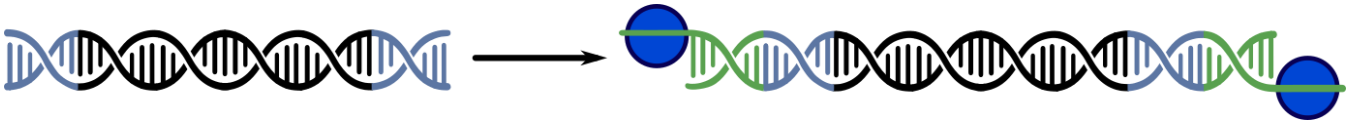
11.5.1.2 Proceed immediately to the next step.

11.6 Remove from the magnet, add 30 µL of water, gently resuspend, and incubate at room temperature for 10 minutes.

11.7 Spin down the sample, pellet on a magnet for 2 minutes or until the supernatant is clear.

11.7.1 Remove and retain the supernatant.

12.0 Adapter Ligation



12.1 Mix:

Table 6: Adapter ligation reaction	
Reagent	**Volume (µL)**
Barcoded, pooled, and cleaned amplicons from previous step	30
NEBNext Quick Ligation Reaction Buffer (5x)	10
Native Adapter (NA)	5
Quick T4 DNA Ligase	5
Total	**50**

12.2 Incubate at room temperature for 20 minutes.

13.0 SPRI Bead Clean Up with Short Fragment Buffer (SFB) Wash and Elution Buffer (EB) Elution

13.1 Bring SPRI beads to room temperature and mix by vortexing.

13.2 Add 0.4x (20 µL) of SPRI beads, mix gently, and incubate at room temperature for 5 minutes.

13.3 Spin down the sample, pellet on a magnet for 2 minutes or until the supernatant is clear.

13.3.1 Remove and discard the supernatant.

13.4 Remove from the magnet, add 125 µL short fragment SFB, flick to resuspend.

13.5 Spin down the sample, pellet on a magnet for 2 minutes or until the supernatant is clear.

13.5.1 Remove and discard the supernatant.

13.6 Remove from the magnet, add 125 µL short fragment SFB, flick to resuspend.

13.7 Spin down the sample, pellet on a magnet for 2 minutes or until the supernatant is clear.

13.7.1 Remove and discard the supernatant.

13.8 Spin down the sample, pellet on a magnet for 10 seconds or until the beads collect to one side.

13.8.1 Remove and discard any residual SFB.

13.9 Remove from the magnet, add 15 µL of EB, gently resuspend, and incubate at room temperature for 10 minutes.

13.10 Spin down the sample, pellet on a magnet for 2 minutes or until the supernatant is clear.

13.10.1 Remove, retain, and quantify the supernatant.

14.0 Prepare the Flow Cell

14.1 If applicable and possible, power cycle the GridION.

14.2 Insert the flow cell and perform a flow cell check.

15.0 Sequencing on Flongle Flow Cells: FLO-FLG114

15.1 Mix the final library using the following calculations and table:

15.1.1 Influenza A amplicon dilution: 5-10 ng of DNA in 5 µL of EB

15.1.2 SARS-CoV-2 amplicon dilutions: 8-15 ng of DNA in 5 µL of EB

15.2 Use SB and LIB from glass vials provided by Flongle Sequencing Expansion kit (EXP-FSE002) that ships with FLO-FLG114.

Table 7: FLO-FLG114 Final Library	
Reagent	**Volume (µL)**
DNA in EB: 5-10 ng (FluA) **or** 8-15 ng (SC2 S-gene)	5
Sequencing Buffer (SB)	15
Resuspended Library Library Beads (LIB)	10
Total	**30**

15.3 Prepare for priming.

15.3.1 Use FCF from the glass vial provided by Flongle Sequencing Expansion kit (EXP-FSE002) that ships with FLO-FLG114.

15.3.2 Prepare the priming mix by adding 3 µL of mixed Flush Tether (FCT) to 117 µL of Flush Buffer (FCF).

15.3.3 Peel back the plastic cover.

15.3.4 Aspirate any storage solution from the tape or outside the loading port.

15.4 Prime with 120 µL of priming mix.

15.4.1 Carefully avoid introducing air bubbles.

15.4.2 Pipette priming mix into the loading port by rolling or slowly depressing.

15.4.3 Remove the pipette tip before air is dispensed into the flow cell.

15.4.4 Do not allow the pipette to draw the solution backwards.

15.5 Load the 30 µL library.

15.5.1 Carefully avoid introducing air bubbles.

15.5.2 Mix the final library by pipetting to resuspend the loading beads.

15.5.3 The library may be loaded by inserting the pipette into the loading port or dropwise onto the loading port

15.5.3.1 Pipette library into the loading port by rolling or slowly depressing.

15.5.3.2 Remove the pipette tip before air is dispensed into the flow cell.

15.5.3.3 Do not allow the pipette to draw the solution backwards.

15.5.4 Gently seal the adhesive plastic over the loading port and vents.

16.0 Sequencing on Standard MinION Flow Cells: FLO-MIN114

16.1 Priming

16.1.1 Prepare the priming mix by adding 30 µL of mixed Flush Tether (FCT) to 1170 µL of Flush Buffer (FCF).

16.1.2 Open the priming port.

16.1.3 Carefully avoid introducing air bubbles.

16.1.4 Prime with 800 µL of priming mix into the priming port > 5 minutes before loading.

16.2 Influenza A amplicon dilution: 10-20 ng of DNA in 5 µL of EB

16.3 SARS-CoV-2 amplicon dilutions: 15-30 ng of DNA in 5 µL of EB

Table 8: FLO-MIN114 final DNA library mixture

Reagent	Volume (µL)
Sequencing Buffer (SB)	37.5
Resuspended Library Library Beads (LIB)	25.5
DNA in EB: 10-20 ng (FluA) **or** 15-30 ng (SC2 Spike)	12
Total	**75**

16.4 Loading

16.4.1 With the priming port still open.

16.4.2 Open the spot-on port.

16.4.3 Add 200 µL of priming mix into the priming port.

16.4.3.1 Carefully avoid introducing air bubbles.

16.4.3.2 Roll or slowly depress the pipette to dispense the priming mix into the flow cell.

16.4.3.3 Remove the pipette tip before air is dispensed into the flow cell.

16.4.3.4 Do not allow the pipette to draw the solution backwards as this will draw air into the now open spot-on port.

16.4.4 Immediately load the 75 µL final library into the spot-on port.

16.4.4.1 Pipette up and down to resuspend the loading beads.

16.4.4.2 In a drop-wise fashion, roll or slowly depress the pipette to dispense the library onto the spot-on port.

16.4.5 Close all ports and lids.

17.0 Starting the Sequencing Run

17.1 No spaces in experiment or sample name.

17.2 Kit Selection: SQK-NBD114-96

17.3 Run options:

17.3.1 FLO-FLG114: 12-24 hours

17.3.2 FLO-MIN114: ~30-45 min/barcode used

17.3.3 Minimum read length:

17.3.3.1 FluSeq: 200 bp

17.3.3.2 SpikeSeq: 1,000 bp

17.4 Analysis:

17.4.1 GridION: Live – super accuracy basecalling enabled

17.4.2 MinION Mk1C: Live – high accuracy basecalling enabled

17.4.2.1 FLO-FLG114: basecalling may lag

17.4.2.2 FLO-MIN114: basecalling will lag significantly

17.4.3 Barcoding - enabled

17.4.3.1 Trim barcodes

17.4.3.2 Require barcodes on both ends

17.5 Output:

17.5.1 Reads per file

17.5.1.1 Mk1C: Default

17.5.1.2 GridION

17.5.1.2.1 FLO-FLG114: 1K reads/file

17.5.1.2.2 FLO-MIN114: 10K reads/file

18.0 Related Procedures

18.1 LP-497 – Multi-segment Reverse Transcription PCR of Influenza A Viruses for Sequencing

18.2 LP-471 – Reverse Transcription-PCR of the SARS-CoV-2 S-gene for Sequencing

19.0 References

19.1 Native barcoding amplicons (with SQK-NBD114.96): Oxford Nanopore Technologies

19.1.1 https://store.nanoporetech.com/us/native-barcoding-kit-96-v14.html

19.2 Ligation sequencing amplicons – Native Barcoding Kit 96 V14 (SQK-NBD114.96)

19.2.1 For MinION

19.2.2 https://community.nanoporetech.com/docs/prepare/library_prep_protocols/ligation-sequencing-amplicons-native-barcoding-v14-sqk-nbd114-96/v/nba_9170_v114_revh_15sep2022

19.3 Ligation sequencing amplicons V14 (SQK-LSK114)

19.3.1 For mixing the final library and flongle loading

19.3.2 https://community.nanoporetech.com/docs/prepare/library_prep_protocols/ligation-sequencing-amplicons-sqk-lsk114/v/acde_9163_v114_revm_29jun2022?devices=flongle

19.4 Priming and loading your flow cell

19.4.1 https://community.nanoporetech.com/nanopore_learning/lessons/priming-and-loading-your-flow-cell

19.5 Biosafety in Microbiological and Biomedical Laboratories (BMBL), current edition