Oct 15, 2021

A SARS-CoV-2 Surveillance Sequencing Protocol Optimized for Oxford Nanopore PromethION

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dx.doi.org/10.17504/protocols.io.butbnwin

Coronavirus Method Development Community | UNC Charlotte COVID WBE

Jessica Schlueter
UNC Charlotte

To identify and monitor SARS-CoV-2 variant evolution, UNC Charlotte has introduced a surveillance sequencing program for the campus and surrounding county area. We have generated a step by step protocol which has been optimized from the "High Throughput Nanopore Sequencing of SARS-CoV-2 Viral Genomes from Patient Samples" (Pater et al 2021, https://doi.org/10.1101/2021.02.09.430478) protocol for preparing SARS-CoV 2 viral genome libraries for next generation sequencing using the Oxford Nanopore PromethION instrument. The protocol is designed to work in a 96-well format throughout. It is intended to produce sufficient sequence for genome assembly to meet standards required by GISAID and NCBI for SARS-CoV-2 sequence submission. The protocol has been modified to achieve maximum yield and includes modifications to address isolate sequencing failures arising from low viral titer in some clinical samples (Cq values below 30). The protocol provides detailed guidelines and steps for working with high Cq value clinical samples, as well as a protocol variation for working in half-reaction volumes throughout to reduce sequencing costs.

DOI

dx.doi.org/10.17504/protocols.io.butbnwin

Jannatul Ferdous, Torri Weathers, Visva Bharati Barua, Erin Stiers, Adam France, Kevin C Lambirth, Cynthia Gibas, Jessica A Schlueter 2021. A SARS-CoV-2 Surveillance Sequencing Protocol Optimized for Oxford Nanopore PromethION. **protocols.io** https://dx.doi.org/10.17504/protocols.io.butbnwin

UNC Charlotte Research and Economic Development
Grant ID: -

SARS-CoV-2, ARTIC, Oxford Nanopore, Promethion

protocol ,

May 06, 2021

Oct 15, 2021

49731

- Wear PPE all the time while doing this protocol.
- Clean the workbench and all the pipettes with 70% Ethanol before using.
- Be very careful while washing the samples with beads in the SPRI clean up, barcoding and adapter ligation section, as a loss of a small amount of beads can lead to a significant decrease in recovered DNA.

Citation: Jannatul Ferdous, Torri Weathers, Visva Bharati Barua, Erin Stiers, Adam France, Kevin C Lambirth, Cynthia Gibas, Jessica A Schlueter A SARS-CoV-2 Surveillance Sequencing Protocol Optimized for Oxford Nanopore PromethION
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cDNA Synthesis
10x RT Buffer (ABI HC cDNA kit; Fisher cat. #4368813)
10x RT Random primers (ABI HC cDNA kit; Fisher cat. #4368813)
25x dNTPs Mix, 100 mM
Multiscribe RT, 50 U/uL (ABI HC cDNA kit; Fisher cat. #4368813)
$MgCl_2$, 50 mM

Artic Amplification
Q5 Reaction buffer (New England Biolabs cat #M0491L)
Q5 High fidelity polymerase (New England Biolabs cat #M0491L)
ARTIC V3 pool 1 diluted to 10uM (IDT DNA, contact your rep)
ARTIC V3 pool 2 diluted to 10uM (IDT DNA, contact your rep)
2.5mM dNTP mix

SPRI clean up
AMPure XP Beads (Beckman Coulter cat #A63881)
80% ethanol fresh
Omega EB (Omega Biotek PD089)

Qubit Quantification
Invitrogen Qubit 1x dsDNA HA Assay kit (Fisher cat #Q33231)

Library End repair
Ultra II end prep reaction buffer
Ultra II end prep reaction enzyme

Sample Barcoding
Native barcoding Expansion (Oxford Nanopore EXP-NBD196)
Ultra II ligation master mix (New England Biolabs cat #E7546L)
Ultra II ligation Enhancer (New England Biolabs cat #E7546L)
Short Fragment Buffer (SFB; Oxford Nanopore EXP-SFB001)
Elution buffer (EB)

Adapter Ligation
Adapter Mix II (AMII; Oxford Nanopore EXP-MRT001 or part of EXP-NBD196)
NEB next quick ligation reaction buffer
Quick T4 DNA ligase (New England Biolabs cat #E6056L)
Short Fragment Buffer (SFB; Oxford Nanopore EXP-SFB001)
Elution buffer (EB; Oxford Nanopore EXP-AUX001)

cDNA Synthesis

1 **Reagents :**
10x Random Primer
25x dNTPs Mix

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10x RT Buffer
50mM MgCl2
MultiScribe RT

Thaw everything except MultiScribe RT at room temperature. Mix each of the tubes by vortexing. After that spin down and place on ice.

2 Prepare master mix 1 (MM1) in a 1.5ml Lobind tube on ice.

Prepare master mix for number of samples+ extra 10 samples for errors. For example, if there are 96 sample , prepare master mix for 96 + 10 = 106 samples. Here is the calculation for 96 samples:

Components	Volume* Number of samples	Master mix
Nuclease free water	4 * 106	424 ul
10x Random Primer	2 * 106	212 ul
25x dNTPs Mix	0.8 * 106	84.8 ul

Divide the Master mix into a strip tube so its easy to add to the 96-well plate.

2.1 For High Cq samples :

Prepare master mix 1 (MM1) in a 1.5ml Lobind tube on ice.

Prepare master mix for (number of samples * 2) + extra 10 samples for errors. For example, if there are 96 sample , prepare master mix for (96*2) + 10 = 202 samples. Here is the calculation for 96 samples:

Components	Volume* Number of samples	Master mix
Nuclease free water	4 * 202	808 ul
10x Random Primer	2 * 202	404 ul
25x dNTPs Mix	0.8 * 202	161.6 ul

Divide the Master mix into a strip tube so its easy to add to the 96-well plate.

2.2 For Reduced cost :

Prepare master mix 1 (MM1) in a 1.5ml Lobind tube on ice.

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Prepare master mix for number of samples + extra 10 samples for errors. For example, if there are 96 sample , prepare master mix for (96/2) + 10 = 58 samples. Here is the calculation for 96 samples:

Components	Volume* Number of samples	Master mix
Nuclease free water	4 * 58	232 ul
10x Random Primer	2 * 58	116 ul
25x dNTPs Mix	0.8 * 58	46.4 ul

Divide the Master mix into a strip tube so its easy to add to the 96-well plate

3 Array 10ul RNA into a 96 well plate on an ice block. Label this plate as "CDNA synthesis".

Add 6.8ul of MM1 into the RNA plate.

3.1 For High Cq samples :

Array 20ul RNA into a 96 well plate on an ice block. Label this plate as "CDNA synthesis".

Add 13.6ul of MM1 into the RNA plate.

3.2 For Reduced cost :

Array 5ul RNA into a 96 well plate on an ice block. Label this plate as "CDNA synthesis".

Add 3.4ul of MM1 into the RNA plate.

4 Seal the plate and mix the solution by quick spin in the plate spinner.

Incubate at **65 °C** for 5 min in the thermal cycler .

5 Prepare master mix 2 (MM2) in a 1.5ml Lobind tube on ice.

Prepare master mix for number of samples + extra 10 samples for errors. For example, if there are 96 sample , prepare master mix for 96 + 10 = 106 samples.

Components	Volume* Number of samples	Master Mix

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10x RT Buffer	2 * 106	212 ul
Multiscribe RT	1 * 106	106 ul
MgCl2	0.2 * 106	21.2 ul

Divide the Master mix into a strip tube so its easy to add to the 96-well plate.

5.1 For High Cq samples :

Prepare master mix 2 (MM2) in a 1.5ml Lobind tube on ice.

Prepare master mix for (number of samples * 2) + extra 10 samples for errors. For example, if there are 96 sample , prepare master mix for 96*2 + 10 = 202samples.

Components	Volume* Number of samples	Master Mix
10x RT Buffer	2 * 202	404 ul
Multiscribe RT	1 * 202	202 ul
MgCl2	0.2 * 202	40.4 ul

Divide the Master mix into a strip tube so its easy to add to the 96-well plate.

5.2 For Reduced cost :

Prepare master mix 2 (MM2) in a 1.5ml Lobind tube on ice.

Prepare master mix for number of samples+ extra 10 samples for errors. For example, if there are 96 sample , prepare master mix for 96 + 10 = 106 samples.

Components	Volume* Number of samples	Master Mix
10x RT Buffer	2 * 58	116 ul
Multiscribe RT	1 * 58	58 ul
MgCl2	0.2 * 58	11.6 ul

Divide the Master mix into a strip tube so its easy to add to the 96-well plate.

6 Remove the RNA plate from the thermal cycler and take out the sealer slowly and carefully so that the solutions in the wells do not make any spill.

Add 3.2 ul of MM2 into the RNA plate .

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6.1 For High Cq samples :

Remove the RNA plate from the thermal cycler and take out the sealer slowly and carefully so that the solutions in the wells do not make any spill.

Add 6.4 ul of MM2 into the RNA plate .

6.2 For Reduced cost :

Remove the RNA plate from the thermal cycler and take out the sealer slowly and carefully so that the solutions in the wells do not make any spill.

Add 1.6 ul of MM2 into the RNA plate .

7 Seal the plate and mix the solution by quick spin in the plate spinner.

Incubate the reactions as follows:

25 °C for 00:10:00
37 °C for 02:00:00
85 °C for 00:05:00
Hold at **4 °C**

8 This plate can be stored at **-20 °C** for long term and at **4 °C** for short term.

Artic Amplification

9 **Reagents :**

5X Q5 Reaction buffer
Q5 High-fidelity polymerase
V3 ARTIC primer pool 1 diluted to 10uM
V3 ARTIC primer pool 2 diluted to 10uM
2.5mM dNTP mix

Remove 5X Q5 Reaction buffer, Q5 High-fidelity polymerase, V3 Artic primer pool 1 and 2,

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dNTP mix from the -20 freezer and place on ice to thaw. Mix each of the tubes by vortexing. After that spin down and place on ice.

10 Prepare 2.5mM dNTP in a 2ml microcentrifuge tube as below:
50ul of dATP
50ul of dCTP
50ul of dTTP
50ul of dGTP
1800ul of nuclease free water
Vortex and spin

Prepare 10uM V3 pool 1 in a 1.5 ml tube as below :
100ul V3 pool 1
900ul Nuclease free water
Vortex and spin

Prepare 10uM V3 pool 2 in a 1.5 ml tube as below :
100ul V3 pool 2
900ul Nuclease free water
Vortex and spin

11 Set up the two PCR plate reactions per sample . ARTIC pool 1 and ARTIC pool 2. This should be two 96 well plates for 96 samples.

Label these plates as "Artic Pool 1" and "Artic Pool 2 ".

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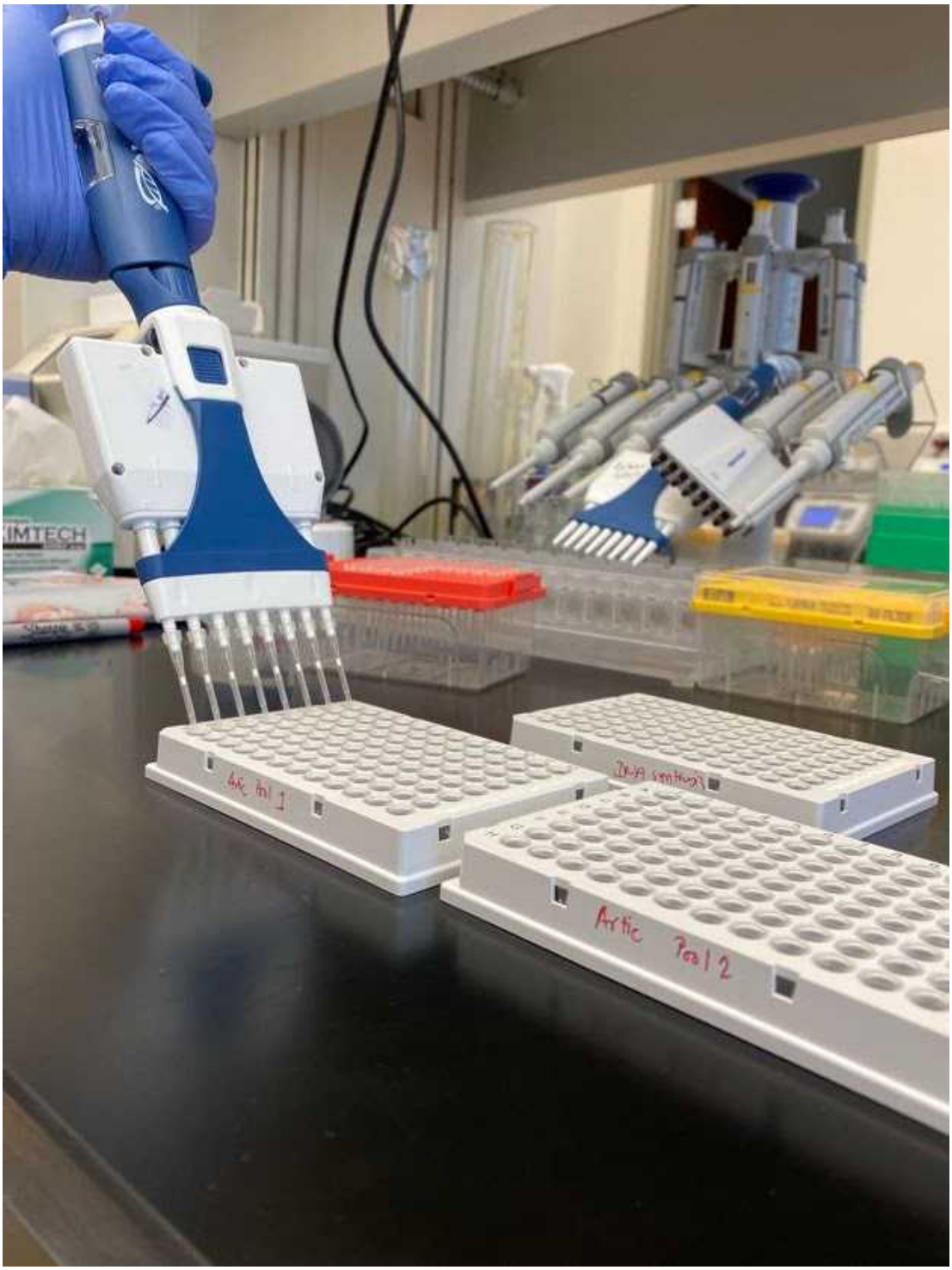

12 Prepare master mix 1 (MM1) into a Falcon tube on ice for ARTIC Pool 1.

Prepare master mix for number of samples + extra 10 samples for errors. For example, if there are 96 sample , prepare master mix for 96 + 10 = 106 samples. Calculations are provided below :

Components	Volume*	Number of samples	Master Mix

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Nuclease free water	8.75 * 106	927.5 ul
Reaction buffer	5 * 106	530 ul
V3 Pool 1	4 * 106	424 ul
2.5mM dNTP mix	2 * 106	212 ul
Q5 High fidelity polymerase	0.25 * 106	26.5 ul

Divide the Master mix into a strip tube so its easy to add to the 96-well plate.

12.1 For High Cq samples :

Prepare master mix 1 (MM1) into a Falcon tube on ice for ARTIC Pool 1.

Prepare master mix for (number of samples*2) + extra 10 samples for errors. For example, if there are 96 sample , prepare master mix for 96*2 + 10 = 202 samples. Calculations are provided below :

Components	Volume* Number of samples	Master Mix
Nuclease free water	8.75 * 202	1767.5 ul
Reaction buffer	5 * 202	1010 ul
V3 Pool 1	4 * 202	808 ul
2.5mM dNTP mix	2 * 202	404 ul
Q5 High fidelity polymerase	0.25 * 202	50.5 ul

Divide the Master mix into a strip tube so its easy to add to the 96-well plate.

12.2 For Reduced cost :

Prepare master mix 1 (MM1) into a Falcon tube on ice for ARTIC Pool 1.

Prepare master mix for number of samples + extra 10 samples for errors. For example, if there are 96 sample , prepare master mix for 96*0.5 + 10 = 58 samples. Calculations are provided below :

Components	Volume* Number of samples	Master Mix
Nuclease free water	8.75 * 58	507.5 ul
Reaction buffer	5 * 58	290 ul
V3 Pool 1	4 * 58	232 ul
2.5mM dNTP mix	2 * 58	116 ul
Q5 High fidelity polymerase	0.25 * 58	14.5 ul

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Divide the Master mix into a strip tube so its easy to add to the 96-well plate.

13 Add 20 ul of MM1 into the ARTIC Pool 1 96 well PCR plate.

Add 5 ul of cDNA into the ARTIC Pool 1 plate.

Seal the plate and mix the solution by quick spin.

13.1 **For High Cq samples :**

Add 40 ul of MM1 into the ARTIC Pool 1 96 well PCR plate.

Add 10 ul of cDNA into the ARTIC Pool 1 plate.

Seal the plate and mix the solution by quick spin.

13.2 **For Reduced cost :**

Add 10 ul of MM1 into the ARTIC Pool 1 96 well PCR plate.

Add 2.5 ul of cDNA into the ARTIC Pool 1 plate.

Seal the plate and mix the solution by quick spin.

14 Prepare master mix 2 (MM2) into a Falcon tube on ice for ARTIC Pool 2.

Prepare master mix for number of samples + extra 10 samples for errors. For example, if there are 96 sample , prepare master mix for 96 + 10 = 106 samples. Calculations are provided below :

Components	Volume* Number of samples	Master Mix
Nuclease free water	8.75 * 106	927.5 ul
Reaction buffer	5 * 106	530 ul
V3 Pool 2	4 * 106	424 ul
2.5mM dNTP mix	2 * 106	212 ul
Q5 High fidelity polymerase	0.25 * 106	26.5 ul

Divide the Master mix into a strip tube so its easy to add to the 96-well plate.

14.1 **For High Cq samples :**

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Prepare master mix for (number of samples*2) + extra 10 samples for errors. For example, if there are 96 sample , prepare master mix for 96*2 + 10 = 202 samples. Calculations are provided below :

Components	Volume* Number of samples	Master Mix
Nuclease free water	8.75 * 202	1767.5 ul
Reaction buffer	5 * 202	1010 ul
V3 Pool 1	4 * 202	808 ul
2.5mM dNTP mix	2 * 202	404 ul
Q5 High fidelity polymerase	0.25 * 202	50.5 ul

Divide the Master mix into a strip tube so its easy to add to the 96-well plate.

14.2 For Reduced cost :

Prepare master mix 1 (MM1) into a Falcon tube on ice for ARTIC Pool 1.

Prepare master mix for number of samples + extra 10 samples for errors. For example, if there are 96 sample , prepare master mix for 96*0.5 + 10 = 58 samples. Calculations are provided below :

Components	Volume* Number of samples	Master Mix
Nuclease free water	8.75 * 58	507.5 ul
Reaction buffer	5 * 58	290 ul
V3 Pool 2	4 * 58	232 ul
2.5mM dNTP mix	2 * 58	116 ul
Q5 High fidelity polymerase	0.25 * 58	14.5 ul

Divide the Master mix into a strip tube so its easy to add to the 96-well plate.

15 Add 20 ul of MM2 into the ARTIC Pool 2 96 well PCR plate.

Add 5 ul of cDNA into the ARTIC Pool 2 plate .

Seal the plate and mix the solution by quick spin.

15.1 For High Cq samples :

Add 40 ul of MM2 into the ARTIC Pool 2 96 well PCR plate.

Add 10 ul of cDNA into the ARTIC Pool 2 plate.

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Seal the plate and mix the solution by quick spin.

15.2 **For Reduced cost :**

Add 10 ul of MM1 into the ARTIC Pool 2 96 well PCR plate.

Add 2.5 ul of cDNA into the ARTIC Pool 2 plate.

Seal the plate and mix the solution by quick spin.

16 Set up the following program on the thermal cycler and put both the ARTIC Pool plates in the cycler .

98 °C	00:00:30	1 cycle
94 °C	00:00:16	20 cycle
65 °C	00:05:00	20 cycle
94 °C	00:00:16	15 cycle
63 °C	00:05:00	15 cycle
Hold at **4 °C**	Indefinite	

17 This plate can be stored at **-20 °C** for long term and at **4 °C** for short term.

SPRI Clean up

18 **Reagents :**

AMPure XP Beads
80% ethanol fresh
Omega EB

19 Prepare 80% ethanol fresh in a 50ml tube as below:

Pour 40 mL 100% ethanol.
Add 10 mL of ddH2O.
Mix by inversion

20 Spin down each PCR product plate of pool 1 and pool 2 at 2,000 RPM for 30 seconds.

Remove the sealer slowly and carefully to avoid contamination.

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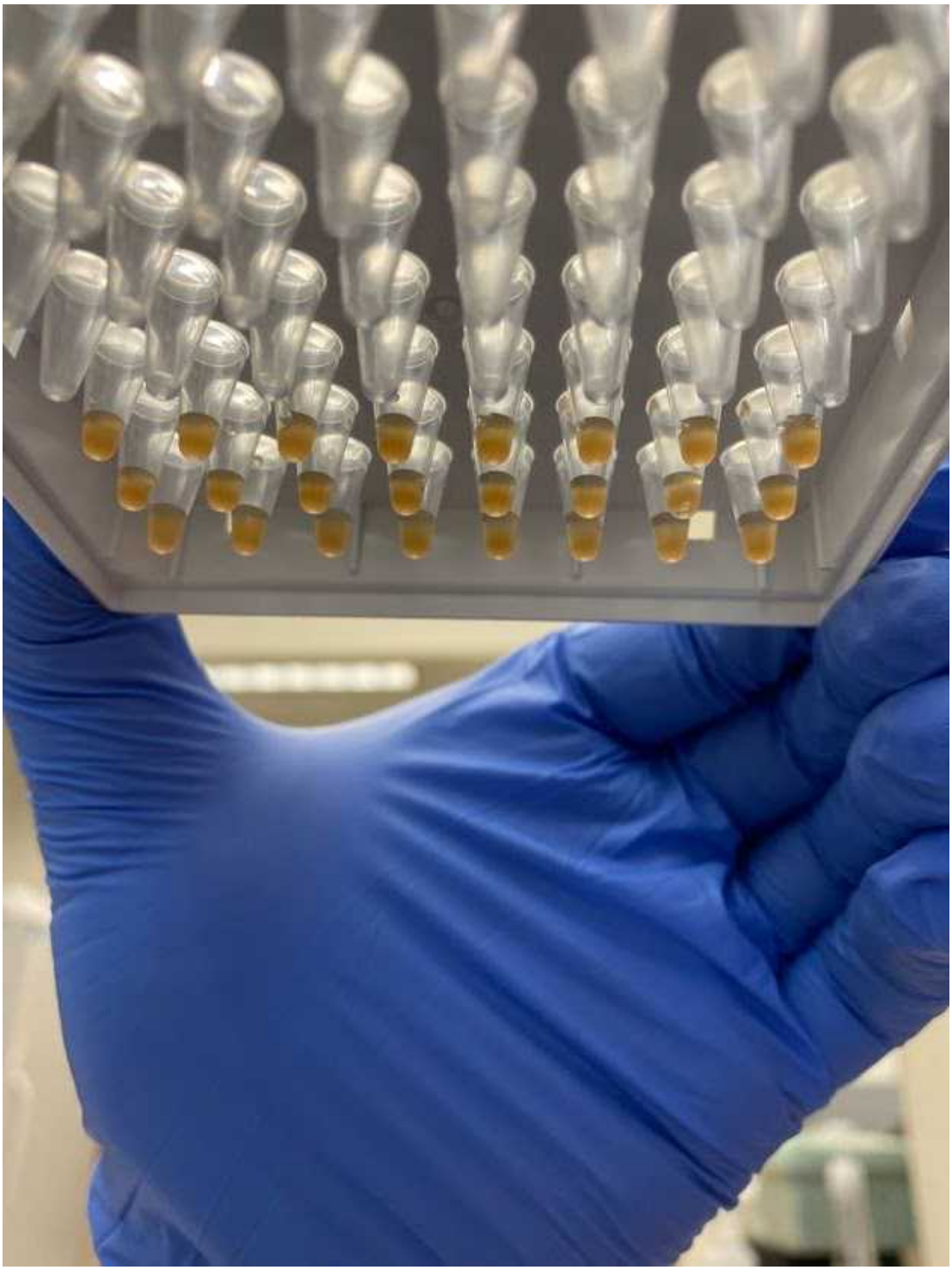
21 Transfer 25ul from column 1 of pool 1 to column 1 of the pool 2 plate. Now column 1 of pool 2 should have a total solution of 50ul. Repeat this until all of the columns of pool 1 have been transferred to pool 2.

Resuspend the stock AMPure XP Beads by vortexing for 15- 20 seconds.

Put 5 mL of AMPure beads into a trough and add 50ul of AMPure beads to each well of the pool 2 plate with an 8-channel pipette. Mix by pipetting up and down 5 times while adding.



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From now on this plate will be called the washing plate.

21.1 For High Cq samples :

Transfer 50ul from column 1 of pool 1 to column 1 of pool 2 plate. Now column 1 of pool 2 should have a total solution of 100ul. Repeat this untill all of of the columns of pool 1 have been tranferred to pool 2.

Put 10 mL of AMPure beads into a trough and add 100ul of AMPure beads to each well of the pool 2 plate with an 8-channel pipette. Mix by pipetting up and down 5 times while adding.

From now on this plate will be called the washing plate.

21.2 For Reduced cost :

Transfer 12.5ul from column 1 of pool 1 to column 1 of pool 2 plate. Now column 1 of pool 2 should have a total solution of 25ul. Repeat this untill all of of the columns of pool 1 have been tranferred to pool 2.

Put 3 mL of AMPure beads into a trough and add 25ul of AMPure beads to each well of the pool 2 plate with an 8-channel pipette. Mix by pipetting up and down 5 times while adding.

From now on this plate will be called the clean-up plate.

22 Cover the 96-well washing plate using a tip box lid. Incubate this plate at room temperature for 15 minutes.

23 After 15 mins, seal the plate with film, spin it for 15seconds and place it on the magnet for 7 minutes.

Check if the solution became clear or not. If not, continue to keep the plate on the magnet.

If clear, carefully discard the supernatant using a P200 multichannel pipette without disturbing the beads.

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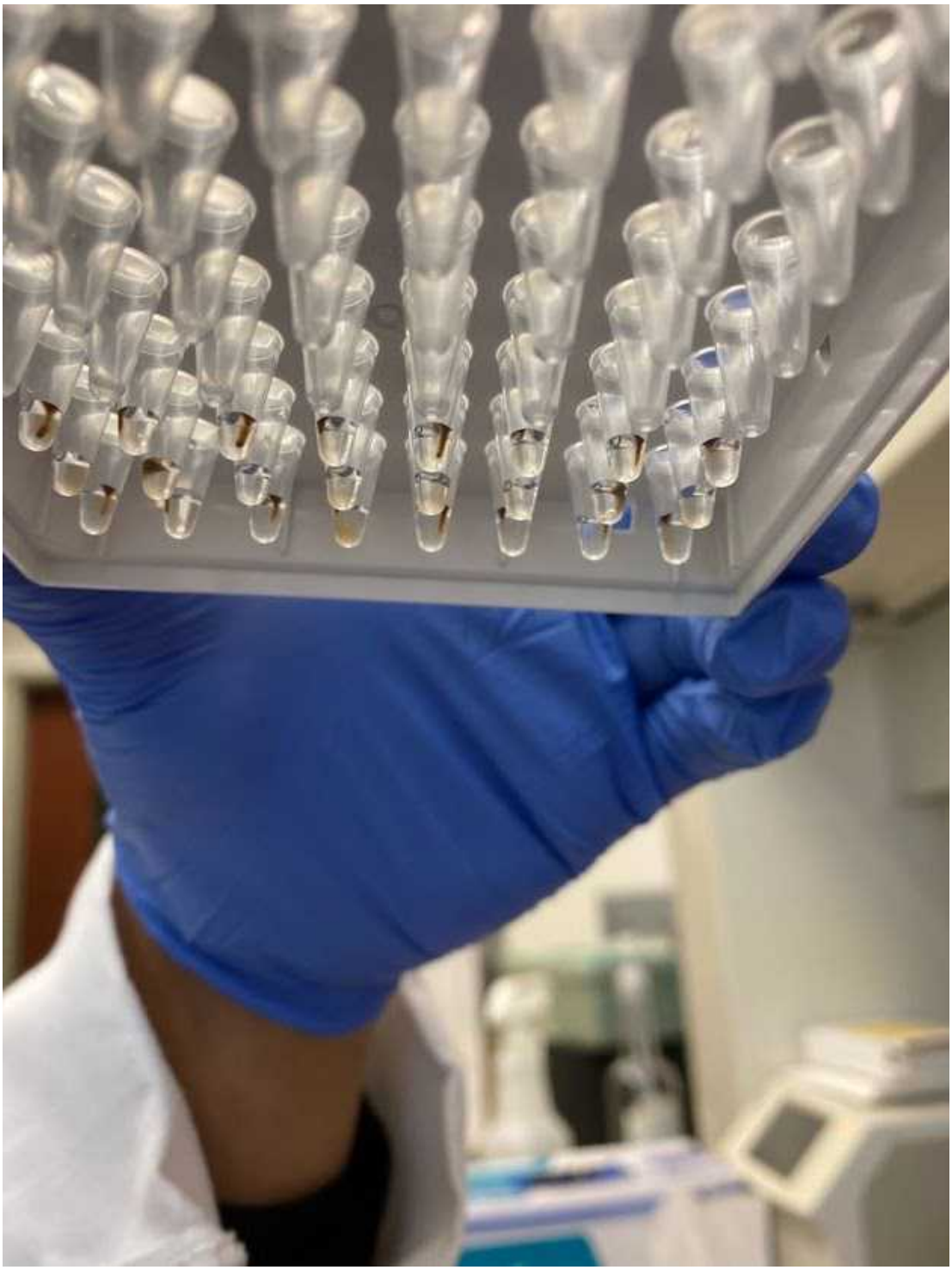

24 While keeping the plate on the magnet, add 200 µL of 80% ethanol to the plate without disturbing the beads.

Wait for 3 minutes with the plate on magnet.

Carefully discard the ethanol using a multichannel pipette while the plate stays on the magnet. This ends the first ethanol wash.

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Repeat this step (80% ethanol wash) for a second ethanol wash.

25 Seal the plate with plate film and spin down the 96-well clean-up plate at 2,000 RPM for 30 seconds.

Place the 96-well clean-up plate on the magnet and remove the plate film.

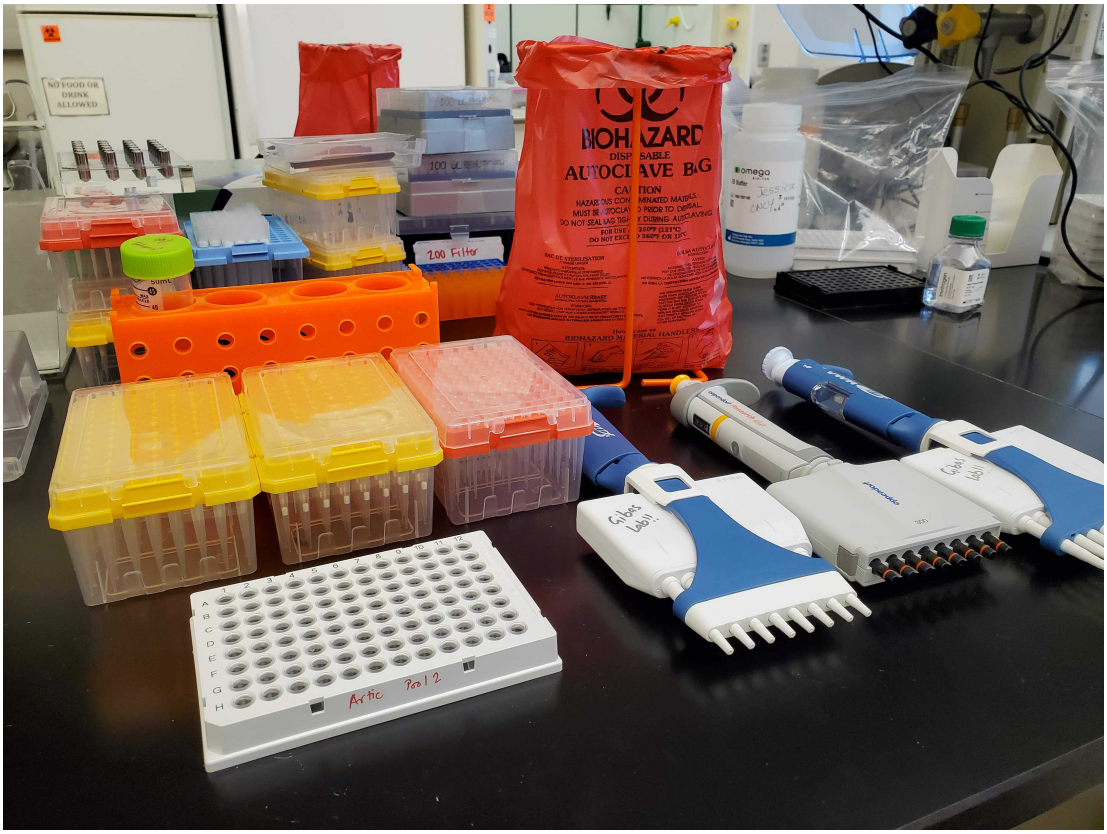
Use a P10 single channel pipette to remove any residual ethanol .

Remove the plate from the magnet and place onto the benchtop.

26 Add 33 µL of Omega EB to each of the wells to elute the DNA and resuspend the beads. Mix by pipetting up and down 5 times.

Remember to change tips and continue until all of the beads in the 96-well clean-up plate have been resuspended.

It is essential to move quickly as the AMPure beads should not dry out, so try to keep everything well set up in the bench before you start resuspending.



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26.1 For High Cq samples :

Add 18 µL of Omega EB to each of the wells to elute the DNA and resuspend the beads. Mix by pipetting up and down 5 times.

Remember to change tips and continue until all of the beads in the 96-well clean-up plate have been resuspended.

It is essential to move quickly as the AMPure beads should not dry out, so try to keep everything well set up in the bench before you start resuspending.

26.2 For Reduced cost :

Add 18 µL of Omega EB to each of the wells to elute the DNA and resuspend the beads. Mix by pipetting up and down 5 times.

Remember to change tips and continue until all of the beads in the 96-well clean-up plate have been resuspended.

It is essential to move quickly as the AMPure beads should not dry out, so try to keep everything well set up in the bench before you start resuspending.

27 Cover the plate using a tip box lid and incubate for 2 minutes at room temperature.

Seal the plate with a plate sealer and spin down the plate containing the resuspended DNA at 2,000 RPM for 15 seconds.

Place the plate on the magnet for 7 minutes.

28 After 7 mins carefully remove and retain 30 µL of the eluate containing the DNA library per well into a new 96-well PCR Plate. Elution with a smaller volume prevents the beads from being eluted as well.

28.1 For High Cq samples :

After 7 mins carefully remove and retain 15 µL of the eluate containing the DNA library per well into a new 96-well PCR Plate. Elution with a smaller volume prevents the beads from being eluted as well.

28.2 For Reduced cost :

After 7 mins carefully remove and retain 15 µL of the eluate containing the DNA library per well into a new 96-well PCR Plate. Elution with a smaller

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volume prevents the beads from being eluted as well.

29 Seal and label the plate containing the eluate as "Clean up plate". This plate will be used for qubit quantification and end-prep reaction.

This plate can be stored at **-20 °C** for long term and at **4 °C** for short term.

Qubit Quantification

30 **Reagents :**
The Qubit quantification kit - comes with 2 standards (standard 1 and standard 2), reagent (dye) and dsDNA HS buffer.

Equilibrate all of the kit components at room temperature.

Note : The qubit quantification kit can also come with prepared qubit solution (dye already added in the HS buffer). In that case no need to prepare the qubit solution separately and move onto step 32.

31 For qubit flex flurometer prepare enough qubit working solution for number of samples and 16 standards (8 standard 1 and 8 standard 2).

For examples if you have 96 samples , prepare solution for - 96 samples + 16 standard + 10 extra = 122 samples.

Prepare Qubit solution in a 50ml tube as follow :

Components	Volume * Number of samples	Solution
dsDNA HS buffer	199 * 122	24278 ul
Qubit dye	1 * 122	122 ul

Mix by solution by vortexing and spin.

Step 31 includes a Step case.
Qubit flurometer (Flex 8-strip tubes)

step case

Qubit flurometer (Flex 8-strip tubes)

Citation: Jannatul Ferdous, Torri Weathers, Visva Bharati Barua, Erin Stiers, Adam France, Kevin C Lambirth, Cynthia Gibas, Jessica A Schlueter A SARS-CoV-2 Surveillance Sequencing Protocol Optimized for Oxford Nanopore PromethION
https://dx.doi.org/10.17504/protocols.io.butbnwin

For qubit flurometer prepare enough qubit working solution for number of samples and 2 standards (1 standard 1 and 1 standard 2).

For examples if you have 96 samples , prepare solution for - 96 samples + 2 standard + 10 extra = 108 samples.

Prepare Qubit solution in a 50ml tube as follow :

Components	Volume * Number of samples	Solution
dsDNA HS buffer	199 * 108	21492 ul
Qubit dye	1 * 108	108 ul

Mix by solution by vortexing and spin.

32 Set up the qubit assay 8 tube strip tubes . One 8-tube strip tube for standard 1, one 8-tube strip tube for standard 2 and twelve 8-tube strip tube for the samples (if you are doing 96 samples).

For standard 1, add 190 ul of qubit working solution to each tube and 10ul of standard 1.
For standard 2, add 190 ul of qubit working solution to each tube and 10ul of standard 2.
For samples, add 199 ul of qubit working solution to each tube and 1ul of sample.

Incubate the standard and samples at room temperature for 2 minutes.
Step 32 includes a Step case.
Qubit flurometer (single tube)

step case

Qubit flurometer (single tube)

Set up the qubit assay tubes . One tube for standard 1, one tube for standard 2 and 96 tube for each of the samples (if you are doing 96 samples).

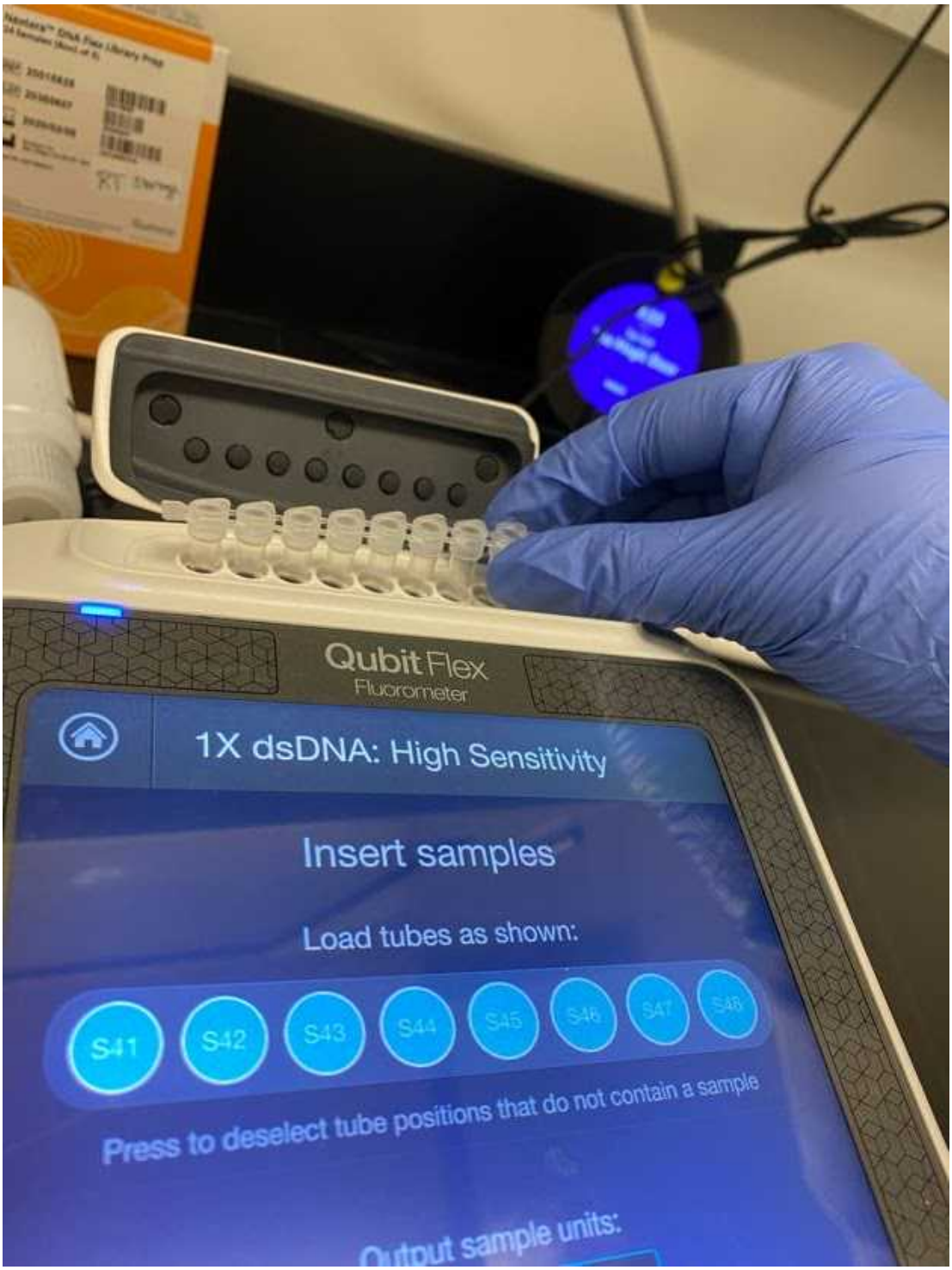
For standard 1, add 190 ul of qubit working solution to the tube and 10ul of standard 1.
For standard 2, add 190 ul of qubit working solution to the tube and 10ul of standard 2.
For samples, add 199 ul of qubit working solution to each tube and 1ul of sample.

Incubate the standard and samples at room temperature for 2 minutes.

33 On the home screen select 1x dsDNA High sensitivity and then select Read new standards and samples.

Insert standard strip tube/single tube in the chamber and close the lid . Remember to put the strip tube in a way so that the left most tubes represent first of the standard 1. Press read standard.

Citation: Jannatul Ferdous, Torri Weathers, Visva Bharati Barua, Erin Stiers, Adam France, Kevin C Lambirth, Cynthia Gibas, Jessica A Schlueter A SARS-CoV-2 Surveillance Sequencing Protocol Optimized for Oxford Nanopore PromethION
https://dx.doi.org/10.17504/protocols.io.butbnwin



Take the tube out and insert standard 2 strip tubes/single tube. Press read standard. This completes the calibration.

Take the reading of the samples the same way as standards. The data of the samples can be retrieved with the qubit flash drive from the section data and then the folder of respective dates.

Citation: Jannatul Ferdous, Torri Weathers, Visva Bharati Barua, Erin Stiers, Adam France, Kevin C Lambirth, Cynthia Gibas, Jessica A Schlueter A SARS-CoV-2 Surveillance Sequencing Protocol Optimized for Oxford Nanopore PromethION
https://dx.doi.org/10.17504/protocols.io.butbnwin

Library End Prep

34 **Reagents :**

NEB Ultra ll end prep reaction buffer
NEB Ultra ll end prep reaction enzyme

Thaw the reagents at room temperature. Mix each of the tubes by vortexing. After that spin down and place on ice.

35 Prepare Master mix in a 1.5ul Lobind tube. If you are doing 96 samples then prepare master mix for number of samples + 10 extra samples. If you are doing 96 samples prepare master mix for 106 samples .

Prepare master mix in a 1.5 tube as follow :

Components	Volume * Number of samples	Master Mix
NEB Ultra ll end prep reaction buffer	2.015 * 106	213.59ul
NEB Ultra ll end prep reaction enzyme	0.8625 * 106	91.425 ul

Divide the Master mix into a strip tube so its easy to add to the 96-well plate.

35.1 **For Reduced cost :**

If you are doing 96 samples then prepare master mix for number of samples*0.5 + 10 extra samples. If you are doing 96 samples prepare master mix for 58 samples .

Prepare master mix in a 1.5 tube as follow :

Components Master Mix	Volume * Number of samples
NEB Ultra ll end prep reaction buffer 116.87ul	2.015 * 58
NEB Ultra ll end prep reaction enzyme 50.025ul	0.8625 * 58

Divide the Master mix into a strip tube so its easy to add to the 96-well plate.

36 Insert the sample DNA concentration from Qubit in the template_sc2_end prep.xlsx spreadsheet , in the DNA (ng/ul) column. This will generate the amount if sample and water needed to be added to normalize all the samples.

Add water according to the mentioned spreadsheet to a new PCR plate labeled as "End prep

Citation: Jannatul Ferdous, Torri Weathers, Visva Bharati Barua, Erin Stiers, Adam France, Kevin C Lambirth, Cynthia Gibas, Jessica A Schlueter A SARS-CoV-2 Surveillance Sequencing Protocol Optimized for Oxford Nanopore PromethION
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plate".

Thaw and spin down the cleaned up PCR plate from SPRI clean up step and add sample according to the spreadsheet from this plate to the new End prep plate.

Add 2.5ul of Mastermix to the End prep plate.

Seal the plate with a plate sealer and spin it down for 15 seconds.

36.1 **For Reduced cost :**

Insert the sample DNA concentration from Qubit in the template_sc2_end prep.xlsx spreadsheet , in the DNA (ng/ul) column. This will generate the amount if sample and water needed to be added to normalize all the samples. Divide the amount of water and sample by 0.5 for each sample. This newly calculated amount of water and sample will be used for normalization.

Add water according to the newly calculated amount to a new PCR plate that we will call end prep plate.

Thaw and spin down the cleaned up PCR plate from SPRI clean up step and add sample according to the newly calculated amount from this plate to the new End prep plate.

Add 1.25ul of Mastermix to the End prep plate.

Seal the plate with a plate sealer and spin it down for 15 seconds.

37 Set up the following program on the thermal cycler and put the End prep plate in the cycler.

20 °C 00:10:00

25 °C 00:05:00

65 °C 00:15:00

Hold at **4 °C** Indefinite

38 Left over clean up plate and finished end prep plate both can be stored at **-20 °C** for long term and at **4 °C** for short term.

Sample Barcoding

39 **Reagents :**

Native barcoding Expansion (EXP - NBD 196)

Citation: Jannatul Ferdous, Torri Weathers, Visva Bharati Barua, Erin Stiers, Adam France, Kevin C Lambirth, Cynthia Gibas, Jessica A Schlueter A SARS-CoV-2 Surveillance Sequencing Protocol Optimized for Oxford Nanopore PromethION
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NEB Ultra ll ligation master mix
NEB Ultra ll ligation Enhancer
ONT Short Fragment Buffer (SFB)
Elution buffer (EB)

Thaw all the reagents at room temperature . Mix each of the tubes by vortexing. After that spin down and place on ice.

40 Prepare the master mix in a 2ml lobind tube. If you are doing 96 samples then prepare master mix for number of samples + 10 extra samples. If you are doing 96 samples prepare master mix for 106 samples .

Prepare master mix in a 1.5 tube as follow :

Components	Volume * Number of samples	Master Mix
Water	5.7 * 106	604.2 ul
NEB Ultra ll ligation master mix	10 * 106	1060 ul
NEB Ultra ll ligation Enhancer	0.3 * 106	31.8 ul

Divide the Master mix into a strip tube so its easy to add to the 96-well plate.

40.1 **For Reduced cost :**

Prepare the master mix in a 1.5ml lobind tube . If you are doing 96 samples then prepare master mix for number of samples*0.5 + 10 extra samples. If you are doing 96 samples prepare master mix for 58 samples .

Prepare master mix in a 1.5 tube as follow :

Components Master Mix	Volume * Number of samples
Water 330.6 ul	5.7 * 58
NEB Ultra ll ligation master mix 580 ul	10 * 58
NEB Ultra ll ligation Enhancer 17.4 ul	0.3 * 58

Divide the Master mix into a strip tube so its easy to add to the 96-well plate.

41 Add 1.5 ul of end prepped DNA in a new 96 well PCR plate. Label this plate as " Barcoding plate".

Citation: Jannatul Ferdous, Torri Weathers, Visva Bharati Barua, Erin Stiers, Adam France, Kevin C Lambirth, Cynthia Gibas, Jessica A Schlueter A SARS-CoV-2 Surveillance Sequencing Protocol Optimized for Oxford Nanopore PromethION
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Add 2.5 ul of barcode in the new barcoding plate.

Add 16 ul of the master mix to the new barcoding plate

Mix all the components by pipetting up and down.

Seal the plate with a plate sealer.

Spin down for 15 seconds in a plate spinner.

Put the leftover end prep plate in **-20 °C**

41.1 **For Reduced cost :**

Add 0.75 ul of end prepped DNA in a new 96 well PCR plate. Label this plate as " Barcoding plate".

Add 1.25 ul of barcode in the new barcoding plate.

Add 8 ul of the master mix to the new barcoding plate

Mix all the components by pipetting up and down.

Seal the plate with a plate sealer.

Spin down for 15 seconds in a plate spinner.

Put the leftover end prep plate in **-20 °C**

42 Set up the following program on the thermal cycler and put the barcoding plate in the cycler .

20 °C 00:20:00
25 °C 00:05:00
65 °C 00:15:00
Hold at **4 °C** Indefinite

43 After the barcoding reaction, take out the plate, remove the seal and pool all the reactions into two 1.5 mL microcentrifuge tube. Split the reaction equally into 2 microcentrifuge tubes.

Mix the contents well and spin down briefly.

For Reduced cost :

Citation: Jannatul Ferdous, Torri Weathers, Visva Bharati Barua, Erin Stiers, Adam France, Kevin C Lambirth, Cynthia Gibas, Jessica A Schlueter A SARS-CoV-2 Surveillance Sequencing Protocol Optimized for Oxford Nanopore PromethION
https://dx.doi.org/10.17504/protocols.io.butbnwin

43.1 After the barcoding reaction, take out the plate, remove the seal and pool all the reactions into a 1.5 mL microcentrifuge tube.

Mix the contents well and spin down briefly.

44 Resuspend the AMPure XP beads by vortexing for 30 seconds.

Check the volume of each microcentrifugre tube and add 0.5x the volume of AMPure XP beads to the each of the tubes.

Mix the beads with sample by pipetting up and down.

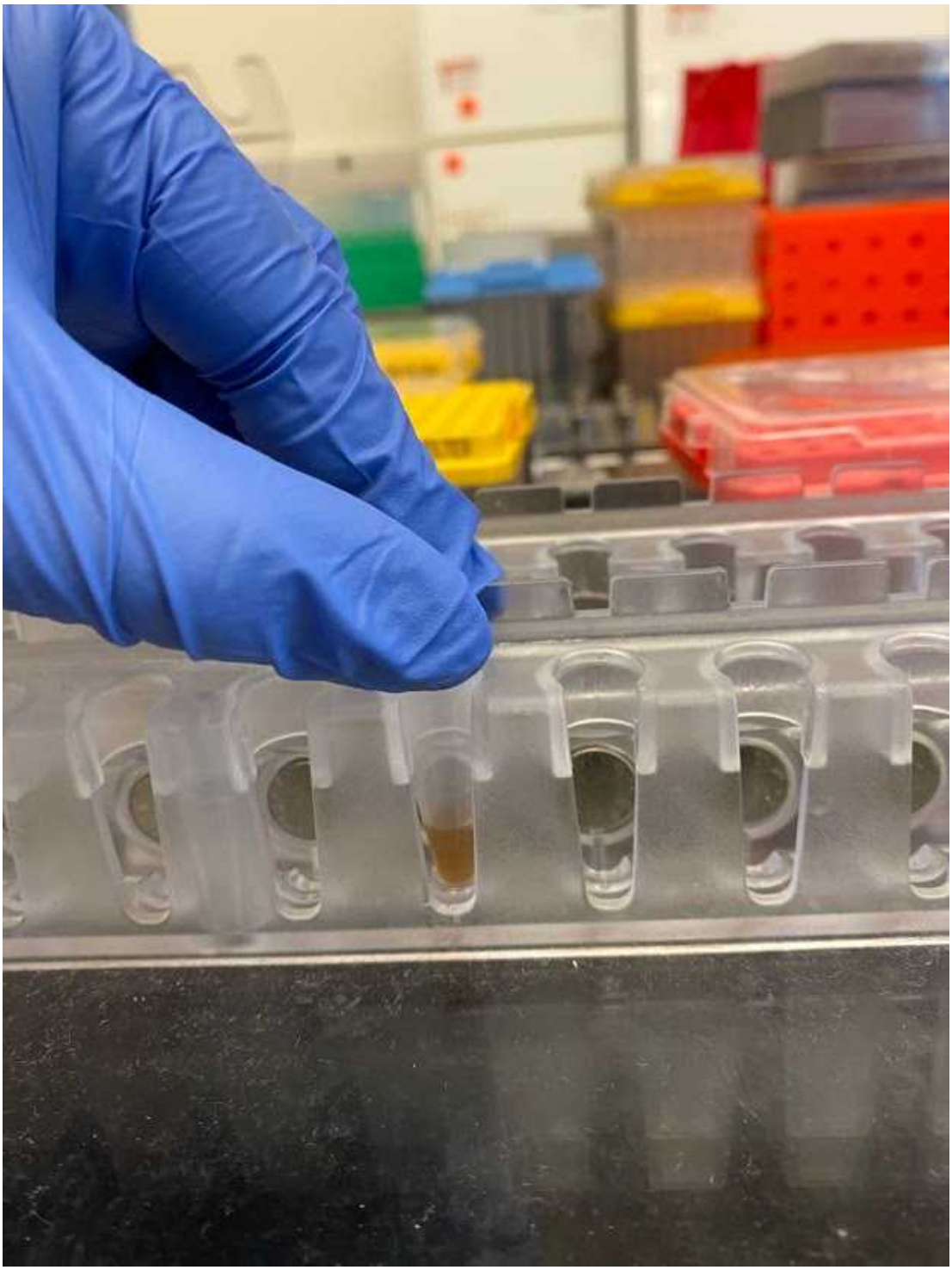
Close the tube tightly and flick the sample several times.

Incubate the tube at room temperature for 10 minutes on a Hula mixer .

45 Take the tubes and spin down for 15 seconds.

Put the tubes in magnetic rack for 7 mins.

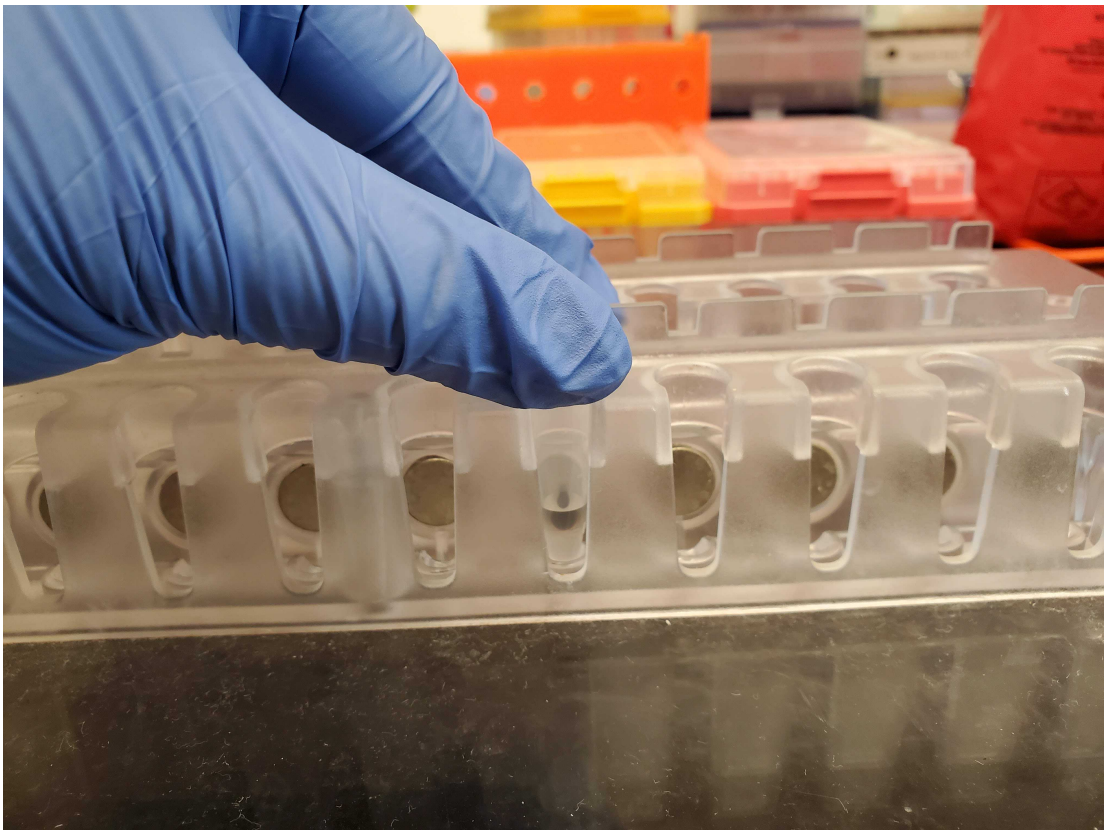
Citation: Jannatul Ferdous, Torri Weathers, Visva Bharati Barua, Erin Stiers, Adam France, Kevin C Lambirth, Cynthia Gibas, Jessica A Schlueter A SARS-CoV-2 Surveillance Sequencing Protocol Optimized for Oxford Nanopore PromethION
https://dx.doi.org/10.17504/protocols.io.butbnwin



Check if the solution became clear or not. If not, continue to keep the plate on the magnet.

If clear, carefully discard the supernatant using a P200 multichannel pipette without disturbing the beads.

Citation: Jannatul Ferdous, Torri Weathers, Visva Bharati Barua, Erin Stiers, Adam France, Kevin C Lambirth, Cynthia Gibas, Jessica A Schlueter A SARS-CoV-2 Surveillance Sequencing Protocol Optimized for Oxford Nanopore PromethION
https://dx.doi.org/10.17504/protocols.io.butbnwin



Remove the tube from the magnet and place it on the benchtop.

46 Add 500 µL of Short Fragment Buffer (SFB) to each of the tubes, pipette up and down and flick the beads to resuspend.

Pulse centrifuge the tubes and place on the magnetic stand for 5 minutes.

Check whether the solution became clear. If not, continue to keep the plate on the magnet.

Keeping the tubes on the magnet, carefully discard the supernatant without disturbing the beads. This ends the first SFB wash.

Repeat this whole step for a second SFB wash.

47 Pulse centrifuge the tubes and place on the magnet.

Use a P10 single channel pipette to remove any residual SFB you observe.

Keeping the tube on the magnet, add 100 µL of freshly made 80% ethanol.

Remove the ethanol, spin down, place on magnet and use a P10 single channel pipette to remove any residual ethanol.

Citation: Jannatul Ferdous, Torri Weathers, Visva Bharati Barua, Erin Stiers, Adam France, Kevin C Lambirth, Cynthia Gibas, Jessica A Schlueter A SARS-CoV-2 Surveillance Sequencing Protocol Optimized for Oxford Nanopore PromethION
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Allow the beads to air dry for ~30 sec-1 min.

48 Remove the tubes from the magnetic stand and place onto the benchtop.

Add 18 µL of the Elution Buffer (EB) to the tube to elute the DNA and resuspend the beads and mix by pipetting up and down.

Incubate at room temperature for 5 minutes.

Flick the tubes several times and pulse centrifuge.

Place the tubes on magnetic stand for 5 minutes.

Take out 15ul clear supernatent from each of the tube.

Pool both of the elutions into a newly labeled 1.5 mL microcentrifuge tube. You should now have 30 µL of pooled barcoded sample.

48.1 **For Reduced cost :**

Remove the tube from the magnetic stand and place onto the benchtop.

Add 18 µL of the Elution Buffer (EB) to the tube to elute the DNA and resuspend the beads and mix by pipetting up and down.

Incubate at room temperature for 5 minutes.

Flick the tubes several times and pulse centrifuge.

Place the tubes on magnetic stand for 5 minutes.

Take out 15ul clear supernatent from the tube into a newly labeled 1.5 mL microcentrifuge tube.

49 Use this pooled sample to do qubit quantification according to the Qubit quantification section. Follow the qubit quantification with qubit flurometer step case.

50 The rest of the pool can be stored at **4 °C** for short term and at **-20 °C** for long term.

Citation: Jannatul Ferdous, Torri Weathers, Visva Bharati Barua, Erin Stiers, Adam France, Kevin C Lambirth, Cynthia Gibas, Jessica A Schlueter A SARS-CoV-2 Surveillance Sequencing Protocol Optimized for Oxford Nanopore PromethION
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Adapter Ligation

51 **Reagents :**

Adapter Mix II
NEB next quick ligation reaction buffer
NEB Quick T4 DNA ligase
Short Fragment Buffer (SFB)
Elution buffer (EB)

Thaw all the reagents at room temperature. Mix each of the tubes by vortexing. After that spin down and place on ice.

52 Thaw the pooled barcoded 30ul sample.

Add 5ul AMII , 5ul Quick T4 DNA ligase and 1oul NEB reaction buffer to the pooled sample.

Mix all the components by pipetting up and down.

Incubate at room temperature for 25 minutes.

52.1 **For Reduced cost :**

Thaw the pooled barcoded 15ul sample.

Add 2.5ul AMII , 2.5ul Quick T4 DNA ligase and 5ul NEB reaction buffer to the pooled sample.

Mix all the components by pipetting up and down.

Incubate at room temperature for 25 minutes.

53 Resuspend the AMPure XP beads by vortexing for 30 seconds.

Add 30ul AMPure XP beads to the pooled sample.

Mix the beads with sample by pipetting up and down.

Close the tube tightly and flick the sample several times.

Incubate the tube at room temperature for 10 minutes on a Hula mixer .

53.1 **For Reduced cost :**

Resuspend the AMPure XP beads by vortexing for 30 seconds.

Citation: Jannatul Ferdous, Torri Weathers, Visva Bharati Barua, Erin Stiers, Adam France, Kevin C Lambirth, Cynthia Gibas, Jessica A Schlueter A SARS-CoV-2 Surveillance Sequencing Protocol Optimized for Oxford Nanopore PromethION
https://dx.doi.org/10.17504/protocols.io.butbnwin

Add 25ul AMPure XP beads to the pooled sample.

Mix the beads with sample by pipetting up and down.

Close the tube tightly and flick the sample several times.

Incubate the tube at room temperature for 10 minutes on a Hula mixer .

54 Take the tube and spin down for 15 seconds.

Put the tubes in magnetic rack for 5 mins.

Check if the solution became clear or not. If not, continue to keep the plate on the magnet.

if clear, carefully discard the supernatant using a P200 multichannel pipette without disturbing the beads.

Remove the tube from the magnet and place it on the benchtop.

55 Add 200 µL of Short Fragment Buffer (SFB) to each of the tubes, pipette up and down and flick the beads to resuspend.

Pulse centrifuge the tubes and place on the magnetic stand for 5 minutes.

Check whether the solution became clear. If not, continue to keep the plate on the magnet.

Keeping the tubes on the magnet, carefully discard the supernatant without disturbing the beads. This ends the first SFB wash.

Repeat this whole process for a second SFB wash.

56 Pulse centrifuge the tubes and place on the magnet.

Use a P10 single channel pipette to remove any residual SFB you observe.

Remove the tubes from the magnetic stand and place onto the benchtop.

57 Add 18 µL of the Elution Buffer (EB) to the tube to elute the DNA and resuspend the beads and mix by pipetting up and down.

Incubate at room temperature for 5 minutes.

Flick the tubes several times and pulse centrifuge.

Citation: Jannatul Ferdous, Torri Weathers, Visva Bharati Barua, Erin Stiers, Adam France, Kevin C Lambirth, Cynthia Gibas, Jessica A Schlueter A SARS-CoV-2 Surveillance Sequencing Protocol Optimized for Oxford Nanopore PromethION
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Place the tubes on magnetic stand for 5 minutes.

Take out 15ul of clear supernatent and put it into a newly labeled 1.5ml tube . This is the final library.

57.1 **For Reduced cost :**

Add 10 µL of the Elution Buffer (EB) to the tube to elute the DNA and resuspend the beads and mix by pipetting up and down.

Incubate at room temperature for 5 minutes.

Flick the tubes several times and pulse centrifuge.

Place the tubes on magnetic stand for 5 minutes.

Take out 7.5ul of clear supernatent and put it into a newly labeled 1.5ml tube . This is the final library.

58 Use this pooled sample to do qubit quantification according to the Qubit quantification section. Follow the qubit quantification with qubit flurometer step case.

59 The rest of the pool can be stored at **4 °C** and at **-20 °C** for long term.

Loading Library Preperation

60 **Reagents :**

Sequencing Buffer (SQB)
Loading buffer (LB)
Flush buffer (FB)
Flush Tether (FLT)

Thaw all the reagents at room temperature . Mix each of the tubes by vortexing. After that spin down and place on ice.

61 Take the last qubit value and multiply by the elution volume of Adapter ligation . Divide the result by 1000. This will be the DNA amount in ug

For example , if the qubit value is 30 then, the DNA amount is (30*15) /1000 = 0.45ug .

61.1 **For Reduced cost :**

Citation: Jannatul Ferdous, Torri Weathers, Visva Bharati Barua, Erin Stiers, Adam France, Kevin C Lambirth, Cynthia Gibas, Jessica A Schlueter A SARS-CoV-2 Surveillance Sequencing Protocol Optimized for Oxford Nanopore PromethION
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Take the last qubit value and multiply by the elution volume of Adapter ligation . Divide the result by 1000. This will be the DNA amount in ug

For example , if the qubit value is 30 then, the DNA amount is (30*7.5) /1000 = 0.225ug .

62 Go to the Biomath calculator. (https://www.promega.com/resources/tools/biomath/) . Insert 400 as DNA length and the calculated DNA amount in previous step in the DNA amount box. Click on calculate. This will give us the pmols of DNA in the sample.

For example, if you give 400 and 0.45 as the input of DNA length and DNA amount in Biomath calculator, it will show that the sample has 1.7pmols of DNA in the sample.

62.1 **For Reduced cost :**

Go to the Biomath calculator. (https://www.promega.com/resources/tools/biomath/) . Insert 400 as DNA length and the calculated DNA amount in previous step in the DNA amount box. Click on calculate. This will give us the pmols of DNA in the sample.

For example, if you give 400 and 0.225 as the input of DNA length and DNA amount in Biomath calculator, it will show that the sample has 0.85pmols of DNA in the sample.

63 Convert the pmol into fmol by multiplying it by 1000. Now divide this fmol by the elution volume to get DNA amount per ul.

For example, the pmol from previous step is 1.7. From there the calculated DNA fmol/ug is (1.7*1000)/15 = 113.33

63.1 **For Reduced cost :**

Convert the pmol into fmol by multiplying it by 1000. Now divide this fmol by the elution volume to get DNA amount per ul.

For example, the pmol from previous step is 0.85. From there the calculated DNA fmol/ug is (0.85*1000)/7.5 = 113.33

64 To prepare the final loading library, find out how much sample is needed to be added in the final library by multiplying it different numbers till it makes the final fmol to 100-105. Make sure not to load less than 100fmol/ul and more than 105fmol/ul. Take the calculated ul of sample in a 1.5ul lobind tube.

After that add water to the sample to make a total solution of 24ul.

Citation: Jannatul Ferdous, Torri Weathers, Visva Bharati Barua, Erin Stiers, Adam France, Kevin C Lambirth, Cynthia Gibas, Jessica A Schlueter A SARS-CoV-2 Surveillance Sequencing Protocol Optimized for Oxford Nanopore PromethION
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Vortex the LB to mix it properly. Add 75ul of SQB and 51ul of LB. Mix by pipetting.

For example : To make it approximately 100fmol/ug , we need to take [(113.33* **0.9**) = 101.99 fmol] 0.9 ul of the library.
Add (24-0.9) =23.1 ul water, 75ul SQB and 51 ul of the LB.

Mix by pipetting.

65 Take out the flow cell from **4 °C** and keep in the room temperature for 25-30 mins and then load them into the docking port and do the flow cell check. Make sure the flow cells click into the port. If the flow cells pass , move on to priming the flow cell. If not, try a different one.

66 Prepare the priming mix by adding 30ul of FLT to a tube if FB. Mix by vortex and spin.

Open the inlet port of the flow cell and draw back a small volume(20-50ul) to remove any air bubble .

Flush 500ul of the priming mix into the inlet port and close the port. Wait for 15 mins.

After 15mins, repeat the priming step with 200ul of priming mix. This ends the priming step .

Citation: Jannatul Ferdous, Torri Weathers, Visva Bharati Barua, Erin Stiers, Adam France, Kevin C Lambirth, Cynthia Gibas, Jessica A Schlueter A SARS-CoV-2 Surveillance Sequencing Protocol Optimized for Oxford Nanopore PromethION
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Add 150ul of the final loading sample library into the inlet port. Close the port.

Wait 10 mins to initiate any experiment in the PromethION.

Citation: Jannatul Ferdous, Torri Weathers, Visva Bharati Barua, Erin Stiers, Adam France, Kevin C Lambirth, Cynthia Gibas, Jessica A Schlueter A SARS-CoV-2 Surveillance Sequencing Protocol Optimized for Oxford Nanopore PromethION
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