PromethION

cDNA-PCR Sequencing V14 - Barcoding (SQK-PCB114.24)

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Kit batch number ..... **Flow cell number** ..... **DNA Samples** .....

Checklist: Reverse transcription and strand-switching

Materials

- [ ] 10 ng enriched RNA (Poly(A)+ RNA or ribodepleted) or 500 ng total RNA per sample
- [ ] cDNA RT Adapter (CRTA)
- [ ] Annealing Buffer (AB)
- [ ] Short Fragment Buffer (SFB)
- [ ] RT Primer (RTP)
- [ ] Strand Switching Primer II (SSPII)

Consumables

- [ ] NEBNext® Quick Ligation Reaction Buffer (NEB, B6058)
- [ ] T4 DNA Ligase 2M U/ml (NEB, M0202M)
- [ ] Lambda Exonuclease (NEB, Cat # M0262L)
- [ ] USER (Uracil-Specific Excision Reagent) Enzyme (NEB, cat # M5505L)
- [ ] Agencourt RNAClean XP beads (Beckman Coulter™, cat # A63987)
- [ ] 10 mM dNTP solution (e.g. NEB cat # N0447)
- [ ] Maxima H Minus Reverse Transcriptase (200 U/µl) with 5x RT Buffer (ThermoFisher, cat # EP0752)
- [ ] RNaseOUT™, 40 U/µl (Life Technologies, cat # 10777019)
- [ ] Qubit RNA HS Assay Kit (ThermoFisher, cat # Q32852)
- [ ] Nuclease-free water (e.g. ThermoFisher, cat #

Equipment

- [ ] Microfuge
- [ ] Thermal cycler
- [ ] Qubit fluorometer (or equivalent for QC check)
- [ ] P1000 pipette and tips
- [ ] P200 pipette and tips
- [ ] P100 pipette and tips
- [ ] P20 pipette and tips
- [ ] P10 pipette and tips
- [ ] P2 pipette and tips

AM9937)

- [ ] 1.5 ml Eppendorf DNA LoBind tubes
- [ ] Qubit™ Assay Tubes (Invitrogen, Q32856)
- [ ] 0.2 ml thin-walled PCR tubes

Reverse transcription and strand-switching

Notes / Observations

CHECKPOINT

Check your flow cell.

We recommend performing a flow cell check before starting your library prep to ensure you have a flow cell with enough pores for a good sequencing run.

See the flow cell check instructions in the MinKNOW protocol for more information.

TIP

Preparing the laboratory for handling RNA samples:

For optimal results, we recommend preparing your laboratory space and equipment prior to handling RNA to ensure the presence of RNAse and contaminants is minimal:

- Clean the lab bench space where you will carry out the work with RNaZap and tech wipes.
- Clean all equipment such as pippettes, tube racks, centrifuge and vortex with RNaZap and tech wipes.
- Use fresh tip boxes and reagents to minimise risk of contamination.

1 Thaw the following reagents, then spin down briefly using a microfuge and mix as indicated in the table below. Then place the reagents on ice.

Reagent	1. Thaw at room temperature	2. Briefly spin down	3. Mix well by pipetting
cDNA RT Adapter (CRTA)	✓	✓	✓
Annealing Buffer (AB)	✓	✓	✓
Short Fragment Buffer (SFB)	✓	✓	✓
RT Primer (RTP)	✓	✓	✓

Reagent	1. Thaw at room temperature	2. Briefly spin down	3. Mix well by pipetting
Strand Switching Primer II (SSPII)	✓	✓	✓
NEBNext® Quick Ligation Reaction Buffer	✓	✓	Mix by vortexing
T4 DNA Ligase 2M U/ml	Not frozen	✓	✓
RNaseOUT	Not frozen	✓	✓
Lambda Exonuclease	Not frozen	✓	✓
Uracil-Specific Excision Reagent (USER)	Not frozen	✓	✓
10 mM dNTP solution	✓	✓	✓
Maxima H Minus Reverse Transcriptase	Not frozen	✓	✓
Maxima H Minus 5x RT Buffer	✓	✓	Mix by vortexing

IMPORTANT

It is important that the NEBNext Quick Ligation Reaction Buffer is mixed well by vortexing.

Check for any visible precipitate; vortexing for at least 30 seconds may be required to solubilise all precipitate.

OPTIONAL ACTION

To run a control experiment, replace your sample input with 10 µl diluted RNA Control Sample (RCS) from the RNA Control Expansion (EXP-RCS001) as follows:

This step differs slightly depending on the concentration of RNA CS (RCS) in your kit. Please ensure you are following the correct method and inputs for your RNA CS (RCS) concentration:

We have increased the concentration of the RNA CS (RCS) vials found in newer batches of EXP-RCS001.

Batch RCS001.10.xxxx or older	**Batch RCS001.20.0001 or newer**
Lower concentration of the RNA CS (RCS) vial:	Increased concentration of the RNA CS (RCS) vial:
15 ng/µl	**50 ng/µl**

- Thaw the RNA Control Sample (RCS) at room temperature, briefly spin down and mix well by pipetting.
- Dilute the RNA Control Sample (RCS) in a 1.5 ml Eppendorf DNA LoBind tube as follows:

For higher concentration RNA CS (RCS): kit batch RCS001.20.0001 or newer:

Reagent	Volume
RNA Control Sample (RCS)	1 µl
Nuclease-free water	46 µl
Total	**47 µl**

Note: This will provide enough volume for 4 samples, adjust your volumes accordingly for the number of samples you wish to run in your control experiment.

- Mix thoroughly by pipetting 10-20 times and briefly spin down.
- Use the **10 µl** of diluted RNA Control Sample (RCS) as your RNA input.

For lower concentration RNA CS (RCS): kit batch RCS001.10.xxxx or older

Reagent	Volume
RNA Control Sample (RCS)	1 µl
Nuclease-free water	14 µl
Total	**15 µl**

Note: This will provide enough volume for 1 sample, adjust your volumes accordingly for the number of samples you wish to run in your control experiment.

- Mix thoroughly by pipetting 10-20 times and briefly spin down.

- Use the **10 µl** of diluted RNA Control Sample (RCS) as your RNA input.

2. For each sample, prepare the RNA in nuclease-free water:
   - Transfer 10 ng Poly(A)+ RNA, or 500 ng total RNA into a 0.2 ml thin-walled PCR tube
   - Adjust the volume to up to 10 µl with nuclease-free water
   - Mix by flicking the tube to avoid unwanted shearing
   - Spin down briefly in a microfuge

3. Prepare the following in a 0.2 ml PCR tube per sample:

Reagent	Volume
RNA	10 µl
cDNA RT Adapter (CRTA)	1 µl
Annealing Buffer (AB)	1 µl
Total volume	**12 µl**

TIP

The cDNA RT Adapter (CRTA) is a double stranded adapter with a poly(T) overhang which anneals to the very end of the poly(A) tail of the RNA strand. This ensures that the full length of the RNA is reverse transcribed and that the poly(A) length can be estimated accurately. Annealing Buffer (AB) has been included to improve CRTA ligation.

4. Mix gently by flicking the tubes, and spin down.

5. Incubate the reactions in the thermal cycler at 60°C for 5 mins, then cool for 5 minutes at room temperature.

6. To each of the 0.2 ml PCR tubes containing you RNA sample(s), add the following:

Reaction	Volume
RNA sample (from previous step)	12 µl
NEBNext® Quick Ligation Reaction Buffer	3.6 µl
T4 DNA Ligase 2M U/ml	1.4 µl
RNaseOUT	1 µl
Total volume (including all reagents)	**18 µl**

7. Ensure the components are thoroughly mixed by pipetting the contents of the tubes 10 times and spin down.

Note: Mix gently to minimise introducing air bubbles to the reactions.

8 Incubate for 10 minutes at room temperature.

9 To each of the 0.2 ml PCR tubes, add the following:

Reagent	Volume
RNA sample (from previous step)	18 µl
Lambda Exonuclease	1 µl
USER (Uracil-Specific Excision Reagent)	1 µl
Total volume (including all reagents)	**20 µl**

TIP

The Lambda Exonuclease and Uracil-Specific Excision Reagent (USER) are third-party reagents used in the preparation of the reverse transcription step. Lambda Exonuclease and USER digest the bottom strand of the ligated CRTA so that the RT Primer (RTP) can bind the CRTA sequence as a primer for the reverse transcription of the RNA.

10 Ensure the components are thoroughly mixed by flicking the tubes and spin down.

11 Incubate for 5 minutes at 37°C in the thermal cycler.

12 Transfer each sample to clean 1.5 ml Eppendorf DNA LoBind tubes.

13 Resuspend the RNase-free XP beads by vortexing.

14 Add 36 µl of resuspended RNase-free XP beads to each reaction and mix gently by flicking the tubes.

15 Incubate on a Hula mixer (rotator mixer) for 5 minutes at room temperature.

16 Spin down the samples and pellet on a magnet. Keep the tubes on the magnet, and pipette off the supernatant.

17 Keep the tubes on the magnet and wash the beads with 100 µl of Short Fragment Buffer (SFB) as follows:

1. Wash the beads with 100 µl of Short Fragment Buffer (SFB).
2. Keeping the magnetic rack on the benchtop, rotate the tube by 180°. Wait for the beads to migrate towards the magnet and to form a pellet.
3. Rotate the tube 180° again (back to the starting position), and wait for the beads to pellet again.
4. Without disturbing the pellet, remove the Short Fragment Buffer (SFB) using a pipette and discard.

18 Repeat the previous step.

19. Spin down and place the tubes back on the magnet. Pipette off any residual buffer. Briefly allow to dry for ~30 seconds, but do not dry the pellet to the point of cracking.

20. Remove the tubes from the magnetic rack and resuspend each pellet in 12 µl of nuclease-free water.

21. Incubate at room temperature for 10 minutes.

22. Pellet the beads on a magnet until the eluate is clear and colourless.

23. Remove and retain 12 µl of eluate into a clean 0.2 ml thin-walled PCR tube per sample.

24. To each of the 0.2 ml PCR tubes, add the following:

Reagents	Volume
Eluted sample (from previous step)	12 µl
RT Primer (RTP)	1 µl
dNTPs (10 mM)	1 µl
Total volume (including all reagents)	**14 µl**

TIP

RT Primer (RTP) is a single stranded primer and binds upstream of the poly(A) tail of the RNA transcript to prime for reverse transcription.

25. Ensure the components are thoroughly mixed by flicking the tubes and spin down.

26. Incubate the reaction for 5 minutes at room temperature.

27. To each of the 0.2 ml PCR tubes, add the following:

Reagents	Volume
RT primed sample (from previous step)	14 µl
Maxima H Minus 5x RT Buffer	4.5 µl
RNaseOUT	1 µl
Strand Switching Primer II (SSPII)	2 µl
Total (including all reagents)	**21.5 µl**

TIP

Strand Switching Primer II (SSPII) base pairs to the deoxycytidine present at the 5' end of the first cDNA strand synthesised. This allows the reverse transcriptase to "strand-switch" for synthesis of the second cDNA strand.

28 Mix gently by flicking the tubes, and spin down.

29 Incubate at 42°C for 2 minutes in the thermal cycler.

30 Add 1 µl of Maxima H Minus Reverse Transcriptase to each tube. The total volume will be 22.5 µl per tube.

31 Mix gently by flicking the tubes, and spin down.

32 Incubate using the following protocol using a thermal cycler:

Cycle step	Temperature	Time	No. of cycles
Reverse transcription and strand-switching	42°C	30 mins	1
Heat inactivation	85°C	5 mins	1
Hold	4°C	∞	

END OF STEP

Take your samples forward into the next step. However, at this point it is also possible to store the sample at -20°C overnight.

Checklist: Selecting for full-length transcripts by PCR

Materials

- [ ] Barcode Primers (BP01-24)
- [ ] Elution Buffer (EB)

Consumables

- [ ] LongAmp Hot Start Taq 2X Master Mix (NEB, M0533)
- [ ] Thermolabile Exonuclease I (NEB, cat # M0568)
- [ ] Agencourt AMPure XP beads (Beckman Coulter™ cat # A63881)
- [ ] Qubit dsDNA HS Assay Kit (Invitrogen, Q32851)
- [ ] Freshly prepared 70% ethanol in nuclease-free water
- [ ] Nuclease-free water (e.g. ThermoFisher, cat # AM9937)
- [ ] 1.5 ml Eppendorf DNA LoBind tubes
- [ ] Qubit™ Assay Tubes (Invitrogen, Q32856)
- [ ] 0.2 ml PCR tubes

Equipment

- [ ] Thermal cycler
- [ ] Vortex mixer
- [ ] Hula mixer (gentle rotator mixer)
- [ ] Magnetic rack, suitable for 1.5 ml Eppendorf tubes
- [ ] Ice bucket with ice
- [ ] P1000 pipette and tips
- [ ] P200 pipette and tips
- [ ] P100 pipette and tips
- [ ] P20 pipette and tips
- [ ] P10 pipette and tips
- [ ] P2 pipette and tips
- [ ] Qubit fluorometer (or equivalent for QC check)
- [ ] Agilent Bioanalyzer (or equivalent)

Selecting for full-length transcripts by PCR

IMPORTANT

This kit enables multiplexing of up to 24 samples. The default method allows you to perform one 25 µl PCR reaction per sample. If multiplexing two or three samples, however, two separate PCR reactions per sample should be performed; if running just one sample, four separate PCR reactions should be performed as per the PCR-cDNA Sequencing Kit V14 (SQK-PCS114) protocol. These recommendations aim to ensure that enough PCR product is generated for optimal flow cell performance.

Reverse transcriptase is a PCR inhibitor and the reverse-transcribed sample must be diluted enough for PCR to take place.

Note: Use one set of Barcode Primers per sample.

Notes / Observations

1. Thaw the following reagents, then spin down briefly using a microfuge and mix as indicated in the table below. Then place the reagents on ice.

Reagent	1. Thaw at room temperature	2. Briefly spin down	3. Mix well by pipetting
Barcode Primers (BP01 - BP24)	✓	✓	✓
Elution Buffer (EB)	✓	✓	✓
LongAmp Hot Start Taq 2X Master Mix	✓	✓	✓
Thermolabile Exonuclease I	Not frozen	✓	✓

2. Spin down the reverse-transcribed RNA samples.

3. Prepare a separate 0.2 ml PCR tube for each sample and add 5 µl of reverse-transcribed RNA per tube.

IMPORTANT

Only 5 µl of the reverse-transcribed sample is to be taken forward. Do NOT use all the 22.5 µl of the reverse transcription reaction in a single PCR reaction.

4. In each of the 0.2 ml PCR tubes containing reverse-transcribed RNA sample, prepare the following reaction at room temperature:

Reagent	Volume
Reverse-transcribed sample (from previous step)	5 µl
Unique Barcode Primer (BP01-24)	0.75 µl
Nuclease-free water	6.75 µl
2x LongAmp Hot Start Taq Master Mix	12.5 µl
Total (including all reagents)	**25 µl**

5. Mix gently by pipetting.

6. Amplify using the following cycling conditions.

Cycle step	Temperature	Time	No. of cycles
Initial denaturation	95°C	30 secs	1
Denaturation	95°C	15 secs	10-18*

Cycle step	Temperature	Time	No. of cycles
Annealing	62°C	15 secs	10-18*
Extension	65°C	60 secs per kb	10-18*
Final extension	65°C	6 mins	1
Hold	4°C	∞	

*We recommend 14 cycles as a starting point. However, the number of cycles can be adjusted between the values shown according to experimental needs.

For further information, please read The effect of varying the number of PCR cycles in the PCR-cDNA Sequencing Kit document.

7. Add 1 µl Thermolabile Exonuclease I directly to each PCR tube. Mix by flicking the tube and briefly spin down.

> TIP
> **The Thermolabile Exonuclease I is added to remove any excess primers which have not successfully annealed.**

8. Incubate the reaction at 37°C for 5 minutes, followed by 80°C for 2 minutes in the thermal cycler.
9. Transfer each sample to a clean 1.5 ml Eppendorf DNA LoBind tube.
10. Resuspend the AMPure XP beads by vortexing.
11. Add 18 µl of resuspended AMPure XP beads to each 1.5 ml Eppendorf DNA LoBind tube.
12. Incubate on a Hula mixer (rotator mixer) for 5 minutes at room temperature.
13. Prepare 5 ml of fresh 70% ethanol in nuclease-free water.
14. Spin down the samples and pellet on a magnet. Keep the tubes on the magnet, and pipette off the supernatant.
15. Keep the tubes on the magnet and wash the beads with 100 µl of freshly-prepared 70% ethanol without disturbing the pellet. Remove the ethanol using a pipette and discard.
16. Repeat the previous step.
17. Spin down and place the tubes back on the magnet. Pipette off any residual ethanol. Allow to dry for ~30 seconds, but do not dry the pellets to the point of cracking.

18 Remove the tubes from the magnetic rack and resuspend each pellet in 12 µl of Elution Buffer (EB).

19 Incubate at room temperature for 10 minutes.

20 Pellet the beads on the magnet until the eluate is clear and colourless.

21 Remove and retain 12 µl of each eluate into a separate clean 1.5 ml Eppendorf DNA LoBind tube.

- Remove and retain the eluate which contains the cDNA library in a clean 1.5 ml Eppendorf DNA LoBind tube
- Dispose of the pelleted beads

22 For each sample, analyse 1 µl of the amplified cDNA for size, quantity and quality using a Qubit fluorometer and Agilent Bioanalyzer (or equivalent) for a QC check.

IMPORTANT

Sometimes a high-molecular weight product is visible in the wells of the gel when the PCR products are run, instead of the expected smear. These libraries are typically associated with poor sequencing performance. We have found that repeating the PCR with fewer cycles can remedy this.

23 Pool together equimolar samples of the amplified cDNA barcoded samples to a total of 50 fmols and make the volume up to 31 µl in Elution Buffer (EB).

Mass	Molarity if fragment length = 0.5 kb	Molarity if fragment length = 1.5 kb	Molarity if fragment length = 3 kb
5 ng	16 fmol	5 fmol	3 fmol
10 ng	32 fmol	11 fmol	5 fmol
15 ng	49 fmol	16 fmol	8 fmol
20 ng	65 fmol	22 fmol	11 fmol
25 ng	81 fmol	27 fmol	13 fmol
50 ng	154 fmol	51 fmol	26 fmol
100 ng	324 fmol	108 fmol	54 fmol

If the quantity of amplified cDNA is above 50 fmol, the remaining cDNA can be frozen and stored for another sequencing experiment (in this case, library preparation would start from the Adapter Addition step). We recommend avoiding multiple freeze-thaw cycles to prevent DNA degradation.

Checklist: Adapter addition

Materials	Consumables	Equipment
☐ Rapid Adapter (RA)	☐ 1.5 ml Eppendorf DNA LoBind tubes	☐ Microfuge
☐ Adapter Buffer (ADB)		☐ Ice bucket with ice
☐ Elution Buffer (EB)		☐ P1000 pipette and tips
		☐ P200 pipette and tips
		☐ P100 pipette and tips
		☐ P20 pipette and tips
		☐ P10 pipette and tips
		☐ P2 pipette and tips

Adapter addition

IMPORTANT

The Rapid Adapter (RA) used in this kit and protocol is not interchangeable with other sequencing adapters.

1. In a fresh 1.5 ml Eppendorf DNA LoBind tube, dilute the Rapid Adapter (RA) as follows and pipette mix:

Reagents	Volume
Rapid Adapter (RA)	1.5 µl
Adapter Buffer (ADB)	3.5 µl
Total	**5 µl**

2. Add 1 µl of the diluted Rapid Adapter (RA) to the amplified cDNA library, making the total volume 32 µl.
3. Mix gently by flicking the tube, and spin down.
4. Incubate the reaction for 5 minutes at room temperature.
5. Spin down briefly.

END OF STEP

The prepared library is used for loading onto the flow cell. Store the library on ice until ready to load.

Notes / Observations

Checklist: Priming and loading the PromethION flow cell

Materials	Consumables	Equipment
☐ Flow Cell Flush (FCF)	☐ PromethION Flow Cell	☐ PromethION 2 Solo device
☐ Flow Cell Tether (FCT)	☐ 1.5 ml Eppendorf DNA LoBind tubes	☐ PromethION sequencing device
☐ Library Solution (LIS)		☐ PromethION Flow Cell Light Shield
☐ Library Beads (LIB)		☐ P1000 pipette and tips
☐ Sequencing Buffer (SB)		☐ P200 pipette and tips
		☐ P20 pipette and tips

Priming and loading the PromethION flow cell	Notes / Observations

IMPORTANT

This kit is only compatible with R10.4.1 flow cells (FLO-PRO114M).

1. Thaw the Sequencing Buffer (SB), Library Beads (LIB) or Library Solution (LIS, if using), Flow Cell Tether (FCT) and Flow Cell Flush (FCF) at room temperature before mixing by vortexing. Then spin down and store on ice.

2. Prepare the flow cell priming mix in a suitable tube for the number of flow cells to flush. Once combined, mix well by briefly vortexing.

Reagent	Volume per flow cell
Flow Cell Tether (FCT)	30 µl
Flow Cell Flush (FCF)	1170 µl
Total volume	**1,200 µl**

IMPORTANT

After taking flow cells out of the fridge, wait 20 minutes before inserting the flow cell into the PromethION for the flow cell to come to room temperature. Condensation can form on the flow cell in humid environments. Inspect the gold connector pins on the top and underside of the flow cell for condensation and wipe off with a lint-free wipe if any is observed. Ensure the heat pad (black pad) is present on the underside of the flow cell.

3. For PromethION 2 Solo, load the flow cell(s) as follows:

    1. Place the flow cell flat on the metal plate.

2. Slide the flow cell into the docking port until the gold pins or green board cannot be seen.

4 For the PromethION 24/48, load the flow cell(s) into the docking ports:

1. Line up the flow cell with the connector horizontally and vertically before smoothly inserting into position.
2. Press down firmly onto the flow cell and ensure the latch engages and clicks into place.

IMPORTANT

Insertion of the flow cells at the wrong angle can cause damage to the pins on the PromethION and affect your sequencing results. If you find the pins on a PromethION position are damaged, please contact support@nanoporetech.com for assistance.

5 Slide the inlet port cover clockwise to open.

IMPORTANT

Take care when drawing back buffer from the flow cell. Do not remove more than 20-30 µl, and make sure that the array of pores are covered by buffer at all times. Introducing air bubbles into the array can irreversibly damage pores.

6 After opening the inlet port, draw back a small volume to remove any air bubbles:

1. Set a P1000 pipette tip to 200 µl.
2. Insert the tip into the inlet port.
3. Turn the wheel until the dial shows 220-230 µl, or until you see a small volume of buffer entering the pipette tip.

7 Load 500 µl of the priming mix into the flow cell via the inlet port, avoiding the introduction of air bubbles. Wait five minutes. During this time, prepare the library for loading using the next steps in the protocol.

8 Thoroughly mix the contents of the Library Beads (LIB) by pipetting.

IMPORTANT

The Library Beads (LIB) tube contains a suspension of beads. These beads settle very quickly. It is vital that they are mixed immediately before use.

We recommend using the Library Beads (LIB) for most sequencing experiments. However, the Library Solution (LIS) is available for more viscous libraries.

9 In a new 1.5 ml Eppendorf DNA LoBind tube, prepare the library for loading as follows:

Reagent	Volume per flow cell
Sequencing Buffer (SB)	100 µl
Library Beads (LIB) thoroughly mixed before use, or Library Solution (LIS)	68 µl
DNA library	32 µl
Total	**200 µl**

Note: Library loading volume has been increased to improve array coverage.

10 Complete the flow cell priming by slowly loading 500 µl of the priming mix into the inlet port.

11 Mix the prepared library gently by pipetting up and down just prior to loading.

12 Load 200 µl of library into the inlet port using a P1000 pipette.

13 Close the valve to seal the inlet port.

IMPORTANT

Install the light shield on your flow cell as soon as library has been loaded for optimal sequencing output.

We recommend leaving the light shield on the flow cell when library is loaded, including during any washing and reloading steps. The shield can be removed when the library has been removed from the flow cell.

14 If the light shield has been removed from the flow cell, install the light shield as follows:

1. Align the inlet port cut out of the light shield with the inlet port cover on the flow cell. The leading edge of the light shield should sit above the flow cell ID.
2. Firmly press the light shield around the inlet port cover. The inlet port clip will click into place underneath the inlet port cover.

END OF STEP

Close the PromethION lid when ready to start a sequencing run on MinKNOW.

Wait a minimum of 10 minutes after loading the flow cells onto the PromethION before initiating any experiments. This will help to increase the sequencing output.

Checklist: Flow cell reuse and returns

Materials	Consumables	Equipment
☐ Flow Cell Wash Kit (EXP-WSH004)		

Flow cell reuse and returns	Notes / Observations

1. After your sequencing experiment is complete, if you would like to reuse the flow cell, please follow the Flow Cell Wash Kit protocol and store the washed flow cell at +2°C to +8°C.

The Flow Cell Wash Kit protocol is available on the Nanopore Community.

2. Alternatively, follow the returns procedure to send the flow cell back to Oxford Nanopore.

Instructions for returning flow cells can be found here.

> **IMPORTANT**
> **If you encounter issues or have questions about your sequencing experiment, please refer to the Troubleshooting Guide that can be found in the online version of this protocol.**