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Version 1

RAPID - RNA Analysis Pipeline for Integrated Diagnostics V.1

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External link: <https://github.com/KylieMontgomery/RAPID>

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Protocol status: Working

We use this protocol and it's working



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Abstract

RAPID is a modular, sample-to-answer workflow for targeted long-read RNA sequencing designed for diagnostic interpretation of splice-altering variants in rare disease. The protocol enables near-full-length transcript analysis from as few as a single sample and a pooled-control, using Oxford Nanopore sequencing. RAPID integrates targeted amplification, sequencing, and analysis to provide reproducible RNA-level evidence supporting variant interpretation, gene exclusion, and downstream DNA investigation, and can be completed within two working days using standard diagnostic laboratory infrastructure. This protocol constitutes the wet-lab component, the dry-lab component can be accessed at <https://github.com/KylieMontgomery/RAPID>.



Materials

RNA collection and extraction

1. **PAXgene® Blood RNA Kit (50 preps)**

PreAnalytiX / QIAGEN

CAT: 762164

2. **PAXgene® Blood RNA Tubes**

PreAnalytiX / QIAGEN

3. **RNeasy® Mini Kit**

QIAGEN

CAT: 74104 (50 preps)

4. **RNase-Free DNase Set (QIAGEN)**

QIAGEN

Reverse transcription and cDNA amplification

1. **NEBNext® Single Cell / Low Input cDNA Synthesis & Amplification Module**

New England Biolabs

CAT: E6421S (24 reactions) / E6421L (96 reactions)

2. **SPRIselect® Reagent**

Beckman Coulter

CAT: B23317

PCR amplification

1. **VeriFi® Hot Start Mix (2×)**

PCR Biosystems

CAT: PB10.46

Nanopore library preparation and sequencing

1. **Native Barcoding Kit 24 V14**

Oxford Nanopore Technologies

CAT: SQK-NBD114.24

2. **NEBNext® Ultra II End Repair / dA-Tailing Module**

New England Biolabs

CAT: E7546

3. **NEB Blunt/TA Ligase Master Mix**

New England Biolabs

CAT: M0367

4. **NEBNext® Quick Ligation Module**

New England Biolabs

CAT: E6056

Quantification and QC

1. **Qubit™ RNA HS Assay Kit**

Thermo Fisher Scientific

CAT: Q32852



2. Qubit™ dsDNA HS Assay Kit

Thermo Fisher Scientific

CAT: Q32851

3. Qubit™ Assay Tubes

Thermo Fisher Scientific

CAT: Q32856

4. Qubit™ Fluorometer

Thermo Fisher Scientific

5. Agilent® Bioanalyzer® or equivalent fragment analyser

Consumables

1. DNA LoBind Tubes (1.5 mL)

Eppendorf

CAT: 022431021

2. DNA LoBind Tubes (2 mL)

Eppendorf

3. Eppendorf twin.tec® PCR plate 96 LoBind, semi-skirted

Eppendorf

CAT: 0030129504

4. DNase/RNase-free PCR strip tubes

USA Scientific

CAT: 1402-1708

5. Nuclease-free water

6. Ethanol (96–100%)

Equipment

1. Thermal cycler

2. Microcentrifuge (1.5–2 mL tubes)

3. Magnetic rack/stand

NEB / Alpaqua or equivalent

CAT: NEB S1515 / Alpaqua A001322

4. Hula mixer / gentle rotator mixer

5. Vortex mixer

Primer design

- 1** Preparation and considerations
It is recommended that GTEX (<https://gtexportal.org/home/gene>) is checked to ensure the gene of interest is sufficiently expressed in the relevant tissue (whole blood, fibroblasts etc) ≥ 1 TPM anything below this is at your discretion. It is also advised to carefully consider specific isoform expression (under exon expression) as the MANE select transcript may not be sufficiently expressed.
- 2** Upload the MANE select fasta (e.g. from NCBI) to Primer-BLAST (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>).
Adjust the 'PCR product size' to allow for the entire transcript to be considered.
Adjust the 'Max target amplicon size' to allow for the entire transcript to be considered.
Under 'Advanced parameters; SNP handling' select 'Primer binding site may not contain known SNP'.
- 3** Check specificity and predicted target length by performing an *in silico* PCR such as that available through the UCSC browser.

RNA extraction - Fibroblasts (Qiagen RNeasy Mini Kit)

51m

- 4** Completely aspirate the cell-culture medium. Add 700 μ l of 1x PBS and aspirate off in order to rinse the cells and proceed immediately to next step. Note: Incomplete removal of cell-culture medium will inhibit lysis and dilute the lysate, affecting the conditions for binding of RNA to the RNeasy membrane. Both effects may reduce RNA yield. 5m
- 5** Disrupt the cells by adding Buffer RLT. For direct lysis of cells grown in a monolayer, add the appropriate volume of Buffer RLT (350 μ l) to the cell-culture dish. Collect the lysate with a rubber policeman. Homogenize by vortexing for 1 min. After homogenization, proceed to step 4. 7m
- 6** Add 1 volume (350 μ l) of 70% ethanol to the homogenized lysate and mix well by pipetting. Important: Do not centrifuge. Note: The volume of lysate may be less than 350 μ l or 700 μ l due to loss during homogenization. Note: When purifying RNA from certain cell lines, precipitates may be visible after addition of ethanol. This does not affect the procedure. 2m
- 7** Transfer up to 700 μ l of the sample, including any precipitate that may have formed, to an RNeasy spin column placed in a 2 ml collection tube. Close the lid gently, and centrifuge for 30 s at $\geq 8000g$ ($\geq 10,000$ rpm). Discard the flow-through.* Reuse the collection tube in future steps. If the sample volume exceeds 700 μ l, centrifuge successive aliquots in the same RNeasy spin column. Discard the flow-through after each centrifugation. 5m



- 8 Add 10 μ l DNase I stock solution to 70 μ l Buffer RDD. Mix by gently inverting the tube. Centrifuge briefly to collect residual liquid from the sides of the tube. Buffer RDD is supplied with the RNase-Free DNase Set. Note: DNase I is especially sensitive to physical denaturation. Mixing should only be carried out by gently inverting the tube. Do not vortex. DNase treatment is not mandatory, it is recommended. 2m
- 9 Add the DNase I incubation mix (80 μ l) directly to the RNeasy spin column membrane, and place on the benchtop (20–30°C) for 15 min. Note: Be sure to add the DNase I incubation mix directly to the RNeasy spin column membrane. DNase digestion will be incomplete if part of the mix sticks to the walls or the O-ring of the spin column. 15m
- 10 Add 350 μ l Buffer RW1 to the RNeasy spin column. Close the lid gently, and centrifuge for 30 s at $\geq 8000g$ ($\geq 10,000$ rpm). Discard the flow-through. 2m
- 11 Add 500 μ l Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 30 s at $\geq 8000g$ ($\geq 10,000$ rpm) to wash the spin column membrane. Discard the flow-through. 2m
- 12 Add 500 μ l Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 2 min at $\geq 8000g$ ($\geq 10,000$ rpm) to wash the spin column membrane. The long centrifugation dries the spin column membrane, ensuring that no ethanol is carried over during RNA elution. Residual ethanol may interfere with downstream reactions. Note: After centrifugation, carefully remove the RNeasy spin column from the collection tube so that the column does not contact the flow-through. Otherwise, carryover of ethanol will occur. 3m
- 13 Place the RNeasy spin column in a new 2 ml collection tube (supplied) and discard the old collection tube with the flow-through. Close the lid gently, and centrifuge at full speed for 1 min. Perform this step to eliminate any possible carryover of Buffer RPE, or if residual flow-through remains on the outside of the RNeasy spin column. 2m
- 14 Place the RNeasy spin column in a new 1.5 ml collection tube (supplied). Add 30 μ l RNase-free water directly to the spin column membrane. Close the lid gently, and centrifuge for 1 min at $\geq 8000 \times g$ ($\geq 10,000$ rpm) to elute the RNA. 2m
- 15 Repeat step 13 using the eluate from step 13 (if high RNA concentration is required). Reuse the collection tube from step 13. If using the eluate from step 13, the RNA yield will be 15–30% less than that obtained using a second volume of RNase-free water, but the final RNA concentration will be higher. 2m
- 16 Transfer 6 μ l of each sample to a 0.2 8-strip tubes for Nanodrop or Qubit and further QC. 2m

RNA extraction - Peripheral blood (Qiagen PAXgene Blood RNA Kit)

1h 26m



- 17 Ensure that the PAXgene Blood RNA Tubes (BRT) are incubated for at least 2 h at room temperature after blood collection to ensure complete lysis of blood cells and precipitation of RNA. Incubation of the PAXgene Blood RNA Tube (BRT) overnight may increase yields. If the initial blood incubation at room temperature for 2 h was not done before storage at 2–8°C, –20°C or –70°C, then first equilibrate the PAXgene Blood RNA Tube (BRT) to room temperature and then incubate it at this temperature for 2 h before starting the procedure. A shaker–incubator is required in steps 5 and 20. Set the temperature of the shaker–incubator to 55°C. Reconstitute stock solutions as needed.
- 18 Centrifuge the PAXgene Blood RNA Tube (BRT) for 10 min at 3000–5000 × g using a swing-bucket rotor. 10m
- 19 Remove the supernatant by decanting or pipetting. Add 4 mL RNase-Free Water (RNFW) to the pellet and close the tube using a fresh secondary BD Hemogard closure (supplied with the kit). 2m
- 20 Vortex until the pellet is visibly dissolved and centrifuge for 10 min at 3000–5000 × g using a swing-bucket rotor. Remove and discard the entire supernatant. 12m
- 21 Add 350 µL resuspension buffer (BR1) and vortex until the pellet is visibly dissolved. 2m
- 22 Pipette the sample into a 1.5 mL MCT. Add 300 µL binding buffer (BR2) and 40 µL proteinase K (PK). Mix by vortexing for 5 s and incubate for 10 min at 55°C using a shaker–incubator at 400–1400 rpm. After incubation, set the temperature of the shaker–incubator to 65°C. 12m
- 23 Pipette the lysate directly into a PSC (lilac) placed in a 2 mL PT and centrifuge for 3 min at maximum speed (but not to exceed 20,000 × g). 5m
- 24 Carefully transfer the entire supernatant of the flow-through fraction to a fresh 1.5 mL MCT without disturbing the pellet in the PT. 1m
- 25 Add 350 µL ethanol (96–100% v/v, purity grade p.a.). Mix by vortexing and centrifuge briefly (1–2 s at 500–1000 × g) to remove drops from the inside of the tube lid. 1m
- 26 Pipette 700 µL sample into the PRC (red) placed in a 2 mL PT and centrifuge for 1 min at 8000–20,000 × g. Place the spin column (PRC) in a new 2 mL PT and discard the old PT containing flow-through. 2m
- 27 Pipette the remaining sample into the PRC and centrifuge for 1 min at 8000–20,000 × g. Place the spin column (PRC) in a new 2 mL PT and discard the 2m



- old PT containing flow-through.
- 28 Pipette 350 μL wash buffer 1 (BR3) into the PRC. Centrifuge for 1 min at 8000–20,000 $\times g$. Place the spin column (PRC) in a new 2 mL PT and discard the old PT containing flow-through. 2m
- 29 Add 10 μL DNase I (RNFD) stock solution to 70 μL DNA digestion buffer (RDD) in a 1.5 mL MCT. Mix by gently flicking the tube and centrifuge briefly to collect residual liquid from the sides of the tube. 1m
- 30 Pipette the DNase I (RNFD) incubation mix (80 μL) directly onto the PRC membrane and place on the benchtop (20–30°C) for 15 min. 15m
- 31 Pipette 350 μL wash buffer 1 (BR3) into the PRC and centrifuge for 1 min at 8000–20,000 $\times g$. Place the spin column (PRC) in a new 2 mL PT and discard the old PT containing flow-through. 2m
- 32 Pipette 500 μL wash buffer 2 (BR4) into the PRC and centrifuge for 1 min at 8000–20,000 $\times g$. Place the spin column (PRC) in a new 2 mL PT and discard the old PT containing flow-through. 2m
- 33 Add another 500 μL wash buffer 2 (BR4) to the PRC. Centrifuge for 3 min at 8000–20,000 $\times g$. 4m
- 34 Discard the PT containing the flow-through and place the PRC in a new 2 mL PT. Centrifuge for 1 min at 8000–20,000 $\times g$. 2m
- 35 Discard the PT containing the flow-through. Place the PRC in a 1.5 mL MCT and pipette 40 μL elution buffer (BR5) directly onto the PRC membrane. Centrifuge for 1 min at 8000–20,000 $\times g$ to elute the RNA. 2m
- 36 Repeat the elution step as described, using 40 μL elution buffer (BR5) and the same MCT. Incubate the eluate for 5 min at 65°C in the shaker–incubator (from step 5) without shaking. After incubation, chill immediately on ice. 7m
- 37 Transfer 6 μL of each sample to a 0.2 8-strip tubes for Nanodrop or Qubit and further QC.

cDNA synthesis (NEBNext®Single Cell/Low Input cDNA Synthesis & Amplification Module)

3h 35m

- 38 Starting Material 2 pg–200 ng poly(A) tail-containing total RNA (DNA free), RIN score $\geq$ 8.0.
Actual yields will depend on the quality and quantity of the input RNA, the mRNA content

of the sample, and the method used to purify the RNA. Typical cDNA yields range between 1-20 ng (for the lower RNA inputs) based on the PCR cycle recommendations.

Pooled RNA control either commercial or in-house should be included as a sample from this point forward and must be of same tissue origin as case samples for analysis.

Keep all buffers and enzymes on ice, unless otherwise indicated.

- 39 To anneal cDNA Primer with total RNA samples, prepare the reaction as follows (on ice):

5m

Component	<5 ng RNA volume (μl) per RXN	≥5 ng RNA volume (μl) per RXN
Total RNA	Up to 8 μl	Up to 7 μl
(Lilac) NEBNext Single Cell RT Primer Mix	1 μl	2 μl
Nuclease-free Water	Variable	Variable
Total Volume	9 μl	9 μl

Mix well by pipetting up and down gently at least 10 times, then centrifuge briefly to collect solution to the bottom of the tubes.

- 40 Incubate for 5 minutes at 70°C in a thermal cycler with the heated lid set to 105°C, then hold at 4°C until next step.

5m

- 41 During the above annealing step, prepare the components for the following step.

5m

Component	Volume (μl) per RXN
(Lilac) NEBNext Single Cell RT Buffer	5 μl
(Lilac) NEBNext Template Switching Oligo	1 μl



Component	Volume (μl) per RXN
(Lilac) NEBNext Single Cell RT Enzyme Mix	2 μl
Nuclease- free Water	3 μl
Total Volume	11 μl

Mix thoroughly by pipetting up and down several times, then centrifuge briefly to collect solutions to the bottom of tubes.

- 42 Combine 11 μl of the RT mix (above) with 9 μl of the annealed sample (Step 2.2.3.). Mix well by pipetting up and down at least 10 times, and centrifuge briefly.

2m

- 43 Incubate the reaction mix in a thermal cycler with the following steps and the heated lid set to 105°C:
90 minutes at 42°C
10 minutes at 70°C
Hold at 4°C

1h 40m

Safe Stopping Point: Samples can be safely stored overnight at 4°C or –20°C.

- 44 Prepare cDNA amplification mix as follows:

5m

Component	Volume (μl) per RXN
(Orange or White) NEBNext Single Cell cDNA PCR Master Mix	50 μl
(Orange) NEBNext Single Cell cDNA PCR Primer	2 μl
(White) NEBNext Cell Lysis Buffer	0.5 μl
Nuclease- free Water	27.5 μl



Component	Volume (μl) per RXN
Total Volume	80 μl

Add 80 μl cDNA amplification mix to 20 μl of the sample and mix by pipetting up and down at least 10 times.

- 45 Incubate the reaction in a thermal cycler with the following PCR cycling conditions and the heated lid set to 105°C:

40m

Cycle step	Temp	Time	Cycles
Initial denaturation	98°C	45 seconds	1
Denaturation	98°C	10 seconds	see table
Annealing	62°C	15 seconds	next page
Extension	72°C	3 minutes	(7-21)*
Final extension	72°C	5 minutes	1
Hold	4°C	∞	

Total RNA	Recommended Number of PCR Cycles
2 pg	20-21
10 pg	17-18
100 pg	14-15
1 ng	10-11
10 ng	8-9
100 ng/200 ng	7-8

Recommended Number of PCR Cycles

Safe Stopping Point: Samples can be safely stored overnight at 4°C or –20°C.



- 46 Allow the NEBNext Bead Reconstitution Buffer and the SPRI® beads (if stored at 4°C) to warm to room temperature for at least 30 minutes before use. Vortex SPRI Beads to resuspend well and prepare fresh 80% ethanol. 2m

Add 60 µl (0.6X of sample volume) resuspended beads to the PCR reaction. Mix well by pipetting up and down at least 10 times. Be careful to expel all of the liquid out of the tip during the last mix. Alternatively, samples can be mixed by vortexing for 3–5 seconds on high. If centrifuging samples after mixing, be sure to stop the centrifugation before the beads start to settle out.

- 47 Incubate samples on the bench top for at least 5 minutes at room temperature. 5m

- 48 Place the tube/ plate on an appropriate magnetic stand to separate the beads from the supernatant. If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells before placing on the magnetic stand. 5m

- 49 After 5 minutes (or when the solution is clear), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain cDNA (Caution: do not discard the beads). 2m

- 50 Add 200 µl of 80% freshly prepared ethanol to the tube/ plate while in the magnetic stand. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant. Be careful not to disturb the beads that contain cDNA. 2m

- 51 Repeat once for a total of two washes. Be sure to remove all visible liquid after the second wash. Briefly spin the tube/ plate, place back on the magnet and remove traces of ethanol with a p10 pipette tip. 3m

- 52 Air dry the beads for 2 minutes (up to 5 minutes) while the tube/ plate is on the magnetic stand with the lid open. 2m

Caution: Do not over-dry the beads. This may result in lower recovery of cDNA. Elute the samples when the beads are still dark brown and glossy looking, but when all visible liquid has evaporated. When the beads turn lighter brown and start to crack they are too dry.

- 53 Remove the tube/ plate from the magnetic stand. Elute the cDNA from the beads by adding 50 µl of 0.1X TE (dilute 1X TE Buffer 1:10 in nuclease-free water). Mix well by pipetting up and down 10 times, or on a vortex mixer. Incubate for at least 2 minutes at room temperature. If necessary, quickly spin the sample to collect the liquid from the sides of the tube. 3m

- 54 Add 45 µl of (room temperature) NEBNext Bead Reconstitution Buffer to the eluted cDNA + bead mixture for a second sample clean up. Mix well by pipetting up and down at least 10 times. Incubate samples on the bench top for at least 5 minutes at room temperature. 6m



(Caution: Skipping this additional cleanup step may reduce overall cDNA purity).

- 55 Place the tube/ plate on an appropriate magnetic stand to separate the beads from the supernatant. If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells before placing on the magnetic stand. After 5 minutes (or when the solution is clear), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain cDNA

6m

(Caution: do not discard the beads).

- 56 Add 200 μ l of 80% freshly prepared ethanol to the tube/ plate while in the magnetic stand. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant. Be careful not to disturb the beads that contain cDNA.

2m

- 57 Repeat once for a total of two washes. Be sure to remove all visible liquid after the second wash. Briefly spin the tube/ plate, place back on the magnet and remove traces of ethanol.

3m

- 58 Air dry the beads for 2 minutes (up to 5 minutes) while the tube/ plate is on the magnetic stand with the lid open.

2m

Caution: Do not over-dry the beads. This may result in lower recovery of cDNA. Elute the samples when the beads are still dark brown and glossy looking, but when all visible liquid has evaporated. When the beads turn lighter brown and start to crack they are too dry.

- 59 Remove the tube/ plate from the magnetic stand. Elute the cDNA from the beads by adding 33 μ l of 1X TE (provided in kit).

1m

(Note: if the downstream library construction protocol recommends that DNA be in solution free of EDTA, elute the cDNA in 10 mM Tris (pH 8.0) instead of TE).

- 60 Mix well by pipetting up and down 10 times, or on a vortex mixer. Incubate for at least 2 minutes at room temperature. If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells before placing back on the magnetic stand.

3m

- 61 Place the tube/ plate on the magnetic stand. After 5 minutes (or when the solution is clear), transfer 30 μ l to a new PCR tube.

6m

Safe Stopping Point: Samples can be safely stored overnight at 4°C or -20°C.

Generation of amplicons (PCR Biosystems VeriFi Hot Start mix)

1h 5m

- 62 Prepare a master mix based on the following table:

5m



Reagent	25 µl RXN	50 µl RXN	Final Concentration
2x VeriFi Hot Start Mix	12.5 µl	25 µl	1x
Forward Primer (10 µM)	1 µl	2 µl	400 nM
Reverse Primer (10 µM)	1 µl	2 µl	400 nM
DMSO	1 µl	2 µl	-
Template cDNA	Up to 100 ng	Up to 200 ng	variable
PCR grade dH ₂ O	Up to 25 µl final volume	Up to 50 µl final volume	

DMSO

63 Cycle using conditions based on the following table:

1h

Cycles	Temp	Time	Notes
1	95°C	1 minute	Initial denatureation
	95°C	15 seconds	Denaturation
25-35	62°C	15 seconds	Annealing
	72°C	10-30 seconds/kb	Extension
	4°C	∞	Final hold

Library preparation (ONT Native Barcoding Kit 24 V14)

2h 35m

64 **End-prep**

15m

In clean 0.2 ml thin-walled PCR tubes (or a clean 96-well plate), aliquot 200 fmol (130 ng for 1 kb amplicons) of DNA per sample. Make up each sample to 11.5 µl using Nuclease-free water. Mix gently by pipetting and spin down.

Combine the following components per tube/well:



11.5 µl 200 fmol (130 ng for 1 kb amplicons) amplicon DNA
1 µl Diluted DNA Control Sample (DCS)
1.75 µl Ultra II End-prep Reaction Buffer
0.75 µl Ultra II End-prep Enzyme Mix

Ensure the components are thoroughly mixed by pipetting and spin down in a centrifuge. Using a thermal cycler, incubate at 20°C for 5 minutes and 65°C for 5 minutes.

65 **Bead clean-up**

30m

Transfer each sample into a clean 1.5 ml Eppendorf DNA LoBind tube. Resuspend the AMPure XP beads (AXP) by vortexing. Add 15 µl of resuspended AMPure XP Beads (AXP) to each end-prep reaction and mix by flicking the tube.

Incubate on a Hula mixer (rotator mixer) for 5 minutes at RT. Prepare 500 µl of fresh 80% ethanol in Nuclease-free water. Spin down the samples and pellet the beads on a magnet until the eluate is clear and colourless. Keep the tubes on the magnet and pipette off the supernatant.

Keep the tube on the magnet and wash the beads with 200 µl of freshly prepared 80% ethanol without disturbing the pellet. Remove the ethanol using a pipette and discard.

Repeat the previous step.

Briefly spin down and place the tubes back on the magnet for the beads to pellet. Pipette off any residual ethanol.

Allow to dry for 30 seconds, but do not dry the pellets to the point of cracking.

Remove the tubes from the magnetic rack and resuspend the pellet in 10 µl Nuclease-free water. Spin down and incubate for 2 minutes at RT. Pellet the beads on a magnet until the eluate is clear and colourless.

Remove and retain 10 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube.

66 **Native barcode ligation**

25m

Select a unique barcode for each sample to be run together on the same flow cell.

In clean 0.2 ml PCR-tubes or a 96-well plate, add the reagents in the following order per well:

7.5 µl End-prepped DNA



2.5 µl Native Barcode (NB01-24)

10 µl Blunt/TA Ligase Master Mix

Thoroughly mix the reaction by gently pipetting and briefly spinning down.

Incubate for 20 minutes at RT.

Add the following volume of EDTA to each well and mix thoroughly by pipetting and spin down briefly.

2 µl For clear cap EDTA

4 µl For blue cap EDTA

Pool all the barcoded samples in a 1.5 ml Eppendorf DNA LoBind tube.

67 **Bead clean-up**

30m

Add 0.4X AMPure XP Beads (AXP) to the pooled reaction, and mix by pipetting.

Incubate on a Hula mixer (rotator mixer) for 10 minutes at RT. Prepare 2 ml of fresh 80% ethanol in Nuclease-free water. Spin down the sample and pellet the beads on a magnet until the eluate is clear and colourless (5 minutes). Keep the tubes on the magnet and pipette off the supernatant.

Keep the tube on the magnet and wash the beads with 700 µl of freshly prepared 80% ethanol without disturbing the pellet. Remove the ethanol using a pipette and discard.

Repeat the previous step.

Briefly spin down and place the tubes back on the magnet for the beads to pellet. Pipette off any residual ethanol.

Allow to dry for 30 seconds, but do not dry the pellets to the point of cracking.

Remove the tubes from the magnetic rack and resuspend the pellet in 35 µl Nuclease-free water, mix by gently flicking. Incubate for 10 minutes at 37°C. Every 2 minutes, agitate the sample by gently flicking for 10 seconds to encourage DNA elution.. Pellet the beads on a magnet until the eluate is clear and colourless.

Remove and retain 35 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube.

68 **Adapter ligation and clean-up**

25m



Depending on the wash buffer (LFB or SFB) used, the clean-up step after adapter ligation is designed to either enrich for DNA fragments of >3 kb, or purify all fragments equally.

To enrich for DNA fragments of 3 kb or longer, use Long Fragment Buffer (LFB)

To retain DNA fragments of all sizes, use Short Fragment Buffer (SFB)

In a 1.5 ml Eppendorf LoBind tube, mix in the following order:

30 µl Pooled barcoded sample

5 µl Native Adapter (NA)

10 µl NEBNext Quick Ligation Reaction Buffer (5X)

5 µl Quick T4 DNA Ligase

Thoroughly mix the reaction by gently pipetting and briefly spinning down.

Incubate the reaction for 20 minutes at RT.

69 **Bead clean-up**

30m

Resuspend the AMPure XP Beads (AXP) by vortexing. Add 20 µl of resuspended AMPure XP Beads (AXP) to the reaction and mix by pipetting.

Incubate on a Hula mixer (rotator mixer) for 10 minutes at RT.

Spin down the sample and pellet on the magnetic rack. Keep the tube on the magnet and pipette off the supernatant.

Wash the beads by adding either 125 µl Long Fragment Buffer (LFB) or Short Fragment Buffer (SFB). Flick

the beads to resuspend, spin down, then return the tube to the magnetic rack and allow the beads to pellet. Remove the supernatant using a pipette and discard.

Repeat the previous step.

Spin down and place the tube back on the magnet. Pipette off any residual supernatant.

Remove the tube from the magnetic rack and resuspend pellet in 15 µl Elution Buffer (EB).

Spin down and incubate for 10 minutes at 37°C. Every 2 minutes, agitate the sample by gently flicking for

10 seconds to encourage DNA elution.

Pellet the beads on a magnet until the eluate is clear and colourless, for at least 1 minute.



Remove and retain 15 μ l of eluate containing the DNA library into a clean 1.5 ml Eppendorf DNA LoBind tube.

Quantify 1 μ l of eluted sample using a Qubit fluorometer. Make up the library to 12 μ l at 10-20 fmol.

IMPORTANT

It is recommended loading 10 - 20 fmol of this final prepared library onto the R10.4.1 flow cell.

Sequencing setup

19m

- 70 To prepare the flow cell priming mix with BSA, combine Flow Cell Flush (FCF) and Flow Cell Tether (FCT), as directed below. Mix by pipetting at RT.

3m

1,170 μ l Flow Cell Flush (FCF)

5 μ l Bovine Serum Albumin (BSA) at 50 mg/ml

30 μ l Flow Cell Tether (FCT)

Open the MinION or GridION device lid and slide the flow cell under the clip. Press down firmly on the flow cell to ensure correct thermal and electrical contact.

Slide the flow cell priming port cover clockwise to open the priming port.

- 71 After opening the priming port, check for a small air bubble under the cover. Draw back a small volume to

6m

remove any bubbles:

Set a P1000 pipette to 200 μ l

Insert the tip into the priming port

Turn the wheel until the dial shows 220-230 μ l, to draw back 20-30 μ l, or until you can see a small volume

of buffer entering the pipette tip

Note: Visually check that there is continuous buffer from the priming port across the sensor array.

Load 800 μ l of the priming mix into the flow cell via the priming port, avoiding the introduction of air bubbles. Wait for five minutes. During this time, prepare the library for loading by following the steps below. Thoroughly mix the contents of the Library Beads (LIB) by pipetting.



- 72 In a new 1.5 ml Eppendorf DNA LoBind tube, prepare the library for loading as follows:
- 37.5 µl Sequencing Buffer (SB)
 - 25.5 µl Library Beads (LIB) mixed immediately before use
 - 12 µl DNA library

5m

Complete the flow cell priming:

Gently lift the SpotON sample port cover to make the SpotON sample port accessible. Load 200 µl of the priming mix into the flow cell priming port (not the SpotON sample port), avoiding the introduction of air bubbles.

Mix the prepared library gently by pipetting up and down just prior to loading. Add 75 µl of the prepared library to the flow cell via the SpotON sample port in a dropwise fashion. Ensure each drop flows into the port before adding the next.

Gently replace the SpotON sample port cover, making sure the bung enters the SpotON port and close the priming port.

- 73 Prior to loading the library for sequencing, ensure sufficient pores are available for sequencing by performing a flow cell check. Flow cell type and ID should be detected by the MinION.

5m

Reduce the kit selection options by selecting sample type 'DNA' and multiplexing 'Yes' then select 'SQK-NBD114-24'.

Run options: Run length is dependant on number of samples multiplex in a single run. Up to 10 should be run for 72 hours and the option to load more library to should considered. For a single sample and pooled control, consider reducing to 4-12 hours to preserve pore life for further sequencing runs. There is the option to adjust to a higher accuracy basecaller, and to output demultiplexed fastq files which will require merging as a single fastq per case or pooled control prior to input into the RAPID pipeline

(<https://github.com/KylieMontgomery/RAPID>).



Protocol references

- **PCR Biosystems.** *VeriFi® Hot Start Mix Instruction Manual (PB10.46)*. PCR Biosystems Ltd.; Version 3.0. PB10.46-VeriFi-Hot-Start-Mix
- **PreAnalytiX GmbH (QIAGEN).** *PAXgene® Blood RNA Kit Handbook*. Version 2; December 2020. HB-0181-005_1122167_HB_PAX_Bloo...
- **QIAGEN.** *RNeasy® Mini Handbook*. Sample to Insight; June 2023. HB-0435-007_HB_RNYMini_0623_WW_...
- **New England Biolabs.** *NEBNext® Single Cell / Low Input cDNA Synthesis & Amplification Module Instruction Manual (E6421)*. Version 6.0; May 2020. manualE6421_1702043936255
- **Oxford Nanopore Technologies.** *Ligation Sequencing Amplicons – Native Barcoding Kit 24 V14 (SQK-NBD114.24)*. Protocol version NBA_9168_v114_revL; September 2022 (last updated October 2023). Ligation sequencing amplicons-Native Barcoding Kit 24 V14 (SQK-NBD114.24)