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Rabies virus MinION sequencing protocol

Forked from Ebola virus sequencing protocol

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1 Works for me dx.doi.org/10.17504/protocols.io.ba4figtn

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ABSTRACT

This is a sample-to-sequence protocol for sequencing rabies virus genomes using the MinION platform (may also be used with Illumina). This pipeline is feasible in low resource settings using a lab-in-a-suitcase approach and has been applied successfully under a range of conditions (from in the field to diagnostic labs) across East Africa, the Philippines and Peru.

The protocol was developed based on resources from the ARTIC network and is published and described in **Rapid in-country sequencing of whole virus genomes to inform rabies elimination programmes (Brunker et al, 2019, Wellcome Open Research).**

GUIDELINES

- Please ensure you keep a good record of sample processing, including a detailed record of sample ids, their specific barcodes, concentrations etc
- Do not rush this protocol! Estimated timings for each step are indicated, based on preparing a batch of 24 samples. These steps may be split across several working days if required, please refer to **Brunker et al, 2019** for advice on timings for each stage and how to divide the workload. Suitable pause points are also indicated in the protocol.

MATERIALS

NAME	CATALOG #	VENDOR
Q5 Hot Start High-Fidelity DNA Polymerase - 500 units	M0493L	New England Biolabs
PCRClean DX	C-1003-5	Aline Biosciences
Filter Tips		
Pipettes		
Mini Centrifuge		
NEBNext Ultra II End Repair/dA-Tailing Module - 96 rxns	E7546L	New England Biolabs
NEBNext Ultra II Ligation Module - 96 rxns	E7595L	New England Biolabs
Nuclease-free water (e.g. MilliQ or HPLC grade water)		
Native Barcoding Kit 1D (EXP-NBD103)		
Invitrogen Quibit or equivalent (We use Promega Quantus Fluorometer) and its corresponding reagents		
Magnetic Rack	View	
Vortex		
LunaScript RT SuperMix Kit	E3010L	New England Biolabs
PCR Machine	View	
DNA LoBind Tubes, 1.5 mL	0030108051	Eppendorf
Ethanol		

NAME	CATALOG #	VENDOR
Custom DNA Oligos, Order Tubes	10693018	Thermo Fisher
DNA AWAY™ Surface Decontaminant, Surface decontaminant; 8.5 oz. (250mL)	7010	Thermo Fisher
ONT MinION Flow Cell R9.4.1	FLO-MIN106D	Oxford Nanopore Technologies
Ligation sequencing kit 1D	SQK-LSK109	Oxford Nanopore Technologies
RNA extraction materials (see protocol in section 1)		
UV lamp (portable/biosafety cabinet)	View	

MATERIALS TEXT

In low-resource settings where standard laboratory equipment is unavailable this protocol can be undertaken using a lab-in-a-suitcase with portable, battery-powered equipment. See the ARTIC network's kit list for a comprehensive list of equipment.

SAFETY WARNINGS

Note that specific safety measures must be undertaken during sample extraction, details can be found in the sub-protocol listed.

BEFORE STARTING

Prepare working area

It is important that the working area is set up appropriately to minimise contamination issues. Separate areas should be designated for sample extraction/template addition, mastermix preparation and post-PCR work. In limited space or low resource settings portable gloveboxes e.g. https://www.amazon.co.uk/Orchid-BO-TBOL-50-50-100-50x50x100-Hydroponics-Hydroponic/dp/B01B1MGRR0 may be used to physically define different areas. These areas should be sterilised with UV and cleaned (details in protocol) between library preparations.

Sample preparation and RNA extraction 3h

1 Extract RNA from brain tissue sample in a dedicated **sample extraction area** (biosafety cabinet/portable glove box)

RNA extraction from field-collected brain tissue samples from suspect rabid animals

by Kirstyn Brunker

PREVIEW RUN

1.1 Brain tissue samples collected in the field may be stored in glycerol-saline, RNA Later or DNA/RNA shield according to the resources available to the sample collector. Instructions to process commonly received samples for use with the **Zymo Research Quick-RNA miniprep kit** are indicated below (for other sample types please refer to the kit instruction manual)

DNase I should be included in the kit (R1054/R1055) but please confirm this is the case before beginning - we have experienced that this is not always the case for certain versions of the kit that may still be in distribution.

1.2 **Homogenised samples stored in DNA/RNA shield**

- Transfer **350 µl** of homogenised sample to a new **2 ml** screw cap tube using a pipette or disposable plastic pastette
- Add **350 µl** of RNA Lysis Buffer (1:1) and mix well

1.3 **Samples stored in RNA later/glycerol-saline**

- Prepare a homogeniser tube by adding 1.4mm ceramic beads (use a 0.2ml PCR tube to measure approx. amount of beads) to a **2 ml** reinforced tube and then add ~ **1 ml** of RNA/DNA shield using a pipette or disposable plastic pastette
- Remove a small piece of tissue* (50-100mg) from RNA later/glycerol using a wooden applicator stick/toothpick/forceps and dab excess liquid on filter paper

*If the sample has liquefied:

- Transfer **200 µl** of liquid to a new 2ml screw cap tube using a pipette or disposable plastic pastette
- Add **200 µl** of RNase-free water or PBS to the sample (1:1). Then add 4 volumes of RNA Lysis Buffer (4:1) and mix.

- Add tissue to the prepared homogeniser tube and ensure the lid is screwed on securely
- Insert tube into the lysis chamber on the Terralyzer and replace chamber shield
- Homogenise the sample for **00:02:00 approx.** and then in **00:00:30 pulses** (if required) until the sample is fully homogenised.

Notes on homogenisation:

- Tissue samples harden in RNA later, therefore may require a longer homogenisation
- If the Terralyzer gets hot, leave to cool for few minutes before using again
- It may be difficult to see if the sample is fully homogenised due to foam- leave so settle for a few minutes and homogenise again if required

- **Leave for 00:02:00 to allow sample inactivation.**
- Transfer **350 µl** of homogenised sample to a new 2ml screw cap tube
- Add **350 µl** of RNA Lysis Buffer (1:1) and mix well.

1.4 RNA extraction and purification is performed using the **Zymo Research Quick-RNA miniprep kit.** The following steps summarise the manufacturer's instructions:

All centrifugation steps should be performed at **10000 x g** - **16000 x g** for **00:00:30** unless otherwise specified.

1.5 Transfer the sample lysed in RNA Lysis Buffer (**700 µl**) into a Spin-Away Filter column (yellow) in a collection tube and centrifuge to remove the majority of genomic DNA. **Save the flow-through.**

To process samples >700 µl, Zymo-Spin columns may be reloaded

1.6 Add a 1:1 volume of ethanol (95-100%) to the sample flow-through and mix well by pipetting up and down

1.7 Transfer the mixture to a Zymo-Spin IIICG column (green) in a collection tube and centrifuge. Discard the flow-through.

1.8 Perform an on-column DNase I treatment:

Prior to use, reconstitute the lyophilized DNase I as indicated on the vial. Store frozen aliquots.

1. Add **400 µl** RNA Wash Buffer to the column and centrifuge. Discard the flow-through.
2. In an RNase-free tube, add **5 µl** DNase I to **75 µl** DNA Digestion Buffer* and mix. Add the mix directly to the column matrix (try not to touch the filter matrix with the pipette tip).
3. Incubate the column at room temperature for **00:15:00**

*If preparing multiple samples make a mastermix

1.9 Add **400 µl** RNA Prep Buffer to the column and centrifuge. Discard the flow- through.

1.10 Add **700 µl** RNA Wash Buffer to the column and centrifuge. Discard the flow-through.

1.11 Add **400 µl** RNA Wash Buffer and centrifuge the column for **00:02:00** to ensure complete removal of the wash buffer. Transfer the column carefully into a **1.5 ml** eppendorf tube (you can discard the collection tube).

1.12 Add **50 µl** DNase/RNase-Free Water directly to the column matrix and centrifuge. **Keep the flow-through: this is the purified RNA!**

The eluted RNA can be used immediately or stored at ≤ **-70 °C** .

cDNA preparation 20m

2 In the **mastermix hood**: prepare a mastermix of the reagents below according to the number of samples and controls to be prepared (plus 1 extra to ensure some excess reagent).
Important: A negative control must be included here and taken right through to sequencing stage.

Component	Volume
LunaScript RT SuperMix (5X)	**2 µl**
Nuclease-free water	**3 µl**

Aliqout 5µL of mastermix for each reaction into labelled 0.2ml PCR strip tubes.

We use half the reaction volume stated in the manufacturer's protocol, using only **2 µl** of Lunascript reagent per **10 µl** reaction and have found this is sufficient to generate enough cDNA.

The Lunascript reagent is quite viscous so take care pipetting, pipette slowly and pause before final purge to ensure reagent is in the tip before dispensing.

3 Take the prepared tubes to the **template hood**. Add **5 µl** of RNA to each tube. Mix gently by pipetting or gently flicking tube and pulse centrifuge to collect liquid at the bottom of the tube. Incubate the reaction as follows on a thermocycler:

25 °C **00:02:00**

55 °C **00:10:00**

95 °C **00:01:00**

Hold at **4 °C**

4 **OPTIONAL PAUSE POINT:** cDNA can be stored at **-20 °C** for up to a month if necessary but it is better to continue to PCR setup if possible.

Primer pool preparation 2h

5 If required resuspend lyophilised primers at a concentration of **100 Micromolar (µM)** each

RABV primers for this protocol were designed using Primal Scheme and generate overlapping 400nt amplicons. There are currently primer schemes available for East Africa, Philippines and Peru, primer sequences are listed in attached documents here:

rabvSEasia_primerSequences.csv **rabvTanzDg_primerSequences.csv**

rabvPeru_primerSequences.csv

6 Generate primer pool stocks by adding **5 µl** of each primer pair to a **1.5 ml** Eppendorf labelled with the primer scheme name and either “Pool 1 (100µM)” or “Pool 2 (100µM)”. These are your **100 Micromolar (µM)** stocks of each primer pool.

Primers should be prepped and aliquoted in a sterile PCR cabinet. At no stage should primers or PCR reagents be anywhere near the template until cDNA addition.

7 Dilute this primer pool 1:10 in molecular grade water, to generate **10 Micromolar (µM)** primer stocks. It is recommend that multiple aliquots of each primer pool are made to in case of degradation or contamination.

Primers need to be used at a final concentration of 0.015µM per primer. For example, for the rabvSEasia scheme, Pool 1 has 42 primers in it so the requirement is 1.575 µL of Pool 1 (10µM) primers per 25µL reaction (i.e. [number of primers X reaction volume (25) x 0.015]/ concentration of primer stock). Pool 2 has 40 primers the requirement is 1.5 µL of Pool 2 (10µM) primers per 25µL reaction. For other schemes, adjust the volume added appropriately.

Multiplex PCR 5h

8 In the mastermix hood prepare a mastermix of the components below according to the number of samples (plus 1 extra) and aliquot **22.5 µl** for each reaction into labelled 0.2mL 8-strip PCR tubes:

Important: Adjust primer volume below according to the scheme used and make up to 22.5ul with water, example shown is for scheme rabvSEasia

Component	Pool 1	Pool 2
NEB Q5® Hot Start Polymerase 2X MasterMix	**12.5 µl**	**12.5 µl**
*Primer Pool 1 or 2 (10µM)	**1.58 µl**	**1.5 µl**
Nuclease-free water	**8.42 µl**	**8.5 µl**
Total	**22.5 µl**	**22.5 µl**

The **mastermix hood** should be prepared by sterilising with UV and treated with MediPal wipes/10% bleach solution, DNAway and RNAseZap reagents. Wipe down the hood with each sequentially, allowing 5 minutes for drying between each. Pipettes should also be treated in the same way, and UV treated for 30 mins between library preparations.

9 In the template hood add **2.5 µl** cDNA to each tube and mix well by pipetting. Pulse centrifuge the tubes to collect the contents at the bottom of the tube.

The **template hood** should prepared by sterilising with UV and treated with MediPal wipes, DNAway and RNAseZap reagents. Wipe down the hood with each sequentially, allowing 5 minutes for drying between each. Pipettes should also be treated in the same way, and UV treated for 30 mins between library preparations.

10 Set-up the following program on the thermal cycler:

Step	Temperature	Time	Cycles
Heat Activation	98 °C	00:00:30	1
Denaturation	98 °C	00:00:15	25-35
Annealing	65 °C	00:05:00	25-35
Hold	4 °C	Indefinite	1

- Cycle number should be 25 for Ct 18-21 up to a maximum of 35 cycles for Ct 35. If you don't have realtime PCR results use 30 cycles!
- Samples do not need to be refrigurated immediately after PCR - in fact the DNA will be stable for a few days at room temperature without suffering from degradation (e.g. see here). Therefore a holding step at 4 °C is not entirely necessary e.g. this temperature may unavailable (e.g. using a MiniPCR machine).

11 **OPTIONAL PAUSE POINT:** PCR products can be stored in the fridge for up to a month or longer term at -20 °C .

PCR clean-up 2h 30m

12 From this point on samples should be prepared in a designated post-PCR area (can be conducted on the bench, if this is separate to mastermix and template areas). Keep the contents of “Pool 1” and “Pool 2” PCR reactions for each biological sample separate.

In future, when you are happy that amplification is relatively equal in each pool, you may want to combine the contents of these tubes for each biological sample prior to clean-up.

13 Clean-up each pool of amplicons using the following protocol:

Amplicon clean-up using SPRI beads
by Josh Quick
PREVIEW RUN

Money saving tips:
- Aline PCRCLEAN DX beads can be used as a direct replacement for Ampure XT beads in this protocol - they cost a lot less!
- In addition, 3-D printed versions of magnetic racks (e.g. tried-and-tested Sam and Tom Industrys Separatron https://samandtomindustrys.science/borchure.html) offer a cheaper alternative to big company versions (just make sure your tubes fit!)

13.1 ⚠

Vortex SPRI beads thoroughly to ensure they are well resuspended, the solution should be a homogenous brown colour.

Agencourt AMPure XP
by Beckman Coulter
Catalog #: A63880

13.2 Add an equal volume (1:1) of SPRI beads to the sample tube and mix gently by either flicking or pipetting. For example add **50 µl** SPRI beads to a **50 µl** reaction.

13.3 Pulse centrifuge to collect all liquid at the bottom of the tube.

13.4 Incubate for **00:05:00** at room temperature.

13.5 Place on magnetic rack and incubate for **00:02:00** or until the beads have pelleted and the supernatant is completely clear.

13.6 Carefully remove and discard the supernatant, being careful not to touch the bead pellet.

13.7 Add **200 µl** of room-temperature [M] **70 % volume** ethanol to the pellet.

13.8 Carefully remove and discard ethanol, being careful not to touch the bead pellet.

13.9 **go to step #7** and repeat ethanol wash.

13.10 Pulse centrifuge to collect all liquid at the bottom of the tube and carefully remove as much residual ethanol as possible using a P10 pipette.

13.11 With the tube lid open incubate for **00:01:00** or until the pellet loses it's shine (if the pellet dries completely it will crack and become difficult to resuspend).

13.12 Resuspend pellet in **30 µl** Elution Buffer (EB), mix gently by either flicking or pipetting and incubate for **00:02:00** .

Elution Buffer (EB)
by Qiagen
Catalog #: 19086

13.13 Place on magnet and transfer sample to a clean 1.5mL Eppendorf tube ensuring no beads are transferred into this tube.

13.14 Quantify **1 µl** product using the Quantus Fluorometer using the ONE dsDNA assay.

QuantiFluor(R) ONE dsDNA System, 100rxn
by Promega
Catalog #: E4871

Quantus
Fluorometer
Promega E6150

14 Quantify the amplicon pools using one of the methods below:

14.1 Quantus Fluorometer with the ONE dsDNA assay

DNA quantification using the Quantus fluorometer
by Josh Quick
PREVIEW RUN

14.1.1 Remove Lambda DNA 400 ng/µL standard from the freezer and leave on ice to thaw. Remove ONE dsDNA dye solution from the fridge and allow to come to room temperature.

QuantiFluor(R) ONE dsDNA System, 500rxn
by Promega
Catalog #: E4870

14.1.2 Set up two **0.5 ml** tubes for the calibration and label them 'Blank' and 'Standard'

14.1.3 Add **200 µl** ONE dsDNA Dye solution to each tube.

14.1.4 Mix the Lambda DNA standard 400 ng/µL standard by pipetting then add **1 µl** to one of the standard tube.

14.1.5 Mix each sample vigorously by vortexing for **00:00:05** and pulse centrifuge to collect the liquid.

14.1.6 Allow both tubes to incubate at room temperature for **00:02:00** before proceeding.

14.1.7 Selection 'Calibrate' then 'ONE DNA' then place the blank sample in the reader then select 'Read Blank'. Now place the standard in the reader and select 'Read Std'.

14.1.8 Set up the required number of **0.5 ml** tubes for the number of DNA samples to be quantified.

Use only thin-wall, clear, 0.5mL PCR tubes such as Axygen #PCR-05-C

14.1.9 Label the tubes on the lids, avoid marking the sides of the tube as this could interfere with the sample reading.

4.1.10 Add **199 µl** ONE dsDNA dye solution to each tube.

4.1.11 Add **1 µl** of each user sample to the appropriate tube.

Use a P2 pipette for highest accuracy.

4.1.12 Mix each sample vigorously by vortexing for **00:00:05** and pulse centrifuge to collect the liquid.

4.1.13 Allow all tubes to incubate at room temperature for **00:02:00** before proceeding.

4.1.14 On the Home screen of the Quantus Fluorometer, select `Protocol`, then select `ONE DNA` as the assay type.

If you have already performed a calibration for the selected assay you can continue, there is no need to perform repeat calibrations when using ONE DNA pre diluted dye solution. If you want to use the previous calibration, skip to step 11. Otherwise, continue with step 9.

4.1.15 On the home screen navigate to 'Sample Volume' and set it to **1 µl** then 'Units' and set it to ng/µL.

4.1.16 Load the first sample into the reader and close the lid. The sample concentration is automatically read when you close the lid.

4.1.17 Repeat step 16 until all samples have been read.

4.1.18 The value displayed on the screen is the dsDNA concentration in ng/µL, carefully record all results in a spreadsheet or laboratory notebook.

14.2 Qubit Fluorometer with the high sensitivity dsDNA assay

DNA quantification using the Qubit fluorometer
by Kirstyn Brunker

PREVIEW RUN

14.2.1 Prepare a mastermix of Qubit™ working solution for the required number of samples and standards. The Qubit dsDNA kit requires 2 standards for calibration (see note below).

Per sample:

Qubit® dsDNA HS Reagent **1 µl**

Qubit® dsDNA HS Buffer **199 µl**

If you have already performed a calibration on the Qubit machine for the selected assay you can use the previous calibration stored on the machine. We recommend performing a new calibration for every sample batch but a same-day calibration would be fine to use for multiple batches.

To avoid any cross-contamination, we recommend that you remove the total amount of working solution required for your samples and standards from the working solution bottle and then add the required volume to the appropriate tubes instead of pipetting directly from the bottle to each tube.

14.2.2 Label the tube lids. Do not label the side of the tube as this could interfere with the sample reading.

Use only thin-wall, clear, 0.5mL PCR tubes. Acceptable tubes include Qubit™ assay tubes (Cat. No. Q32856)

14.2.3 Aliquot Qubit™ working solution to each tube:
- standard tubes requires 190µL of Qubit™ working solution
- sample tubes require anywhere from 180–199µL (depending how much sample you wish to add).

The final volume in each tube must be 200µL once sample/standard has been added.

14.2.4 Add 10µL of standard to the appropriate tube.

14.2.5 Add 1–20µL of each user sample to the appropriate tube.

If you are adding 1–2µL of sample, use a P-2 pipette for best results.

14.2.6 Mix each tube vigorously by vortexing for 3–5 seconds.

14.2.7 Allow all tubes to incubate at room temperature for 2 minutes, then proceed to “Read standards and samples”.

14.2.8 On the Home screen of the Qubit™ 3 Fluorometer, press DNA, then select 1X dsDNA HS as the assay type. The Read standards screen is displayed. Press Read Standards to proceed.

If you have already performed a calibration for the selected assay, the instrument prompts you to choose between reading new standards and running samples using the previous calibration. **If you want to use the previous calibration, skip to step 12**. Otherwise, continue with step 9.

14.2.9 Insert the tube containing Standard #1 into the sample chamber, close the lid, then press Read standard. When the reading is complete (~3 seconds), remove Standard #1.

4.2.10 Insert the tube containing Standard #2 into the sample chamber, close the lid, then press Read standard. When the reading is complete, remove Standard #2.

4.2.11 The instrument displays the results on the Read standard screen. For information on interpreting the calibration results, refer to the Qubit™ Fluorometer User Guide, available for download at thermofisher.com/qubit.

4.2.12 Press Run samples.

4.2.13 On the assay screen, select the sample volume and units:
- Press the + or – buttons on the wheel, or anywhere on the wheel itself, to select the sample volume added to the assay tube (from 1–20µL).
- From the unit dropdown menu, select the units for the output sample concentration (in this case choose ng/µL).

4.2.14 Insert a sample tube into the sample chamber, close the lid, then press Read tube. When the reading is complete (~3 seconds), remove the sample tube.

4.2.15 **The top value (in large font) is the concentration of the original sample and the bottom value is the dilution concentration**. For information on interpreting the sample results, refer to the Qubit™ Fluorometer User Guide.

4.2.16 Repeat step 14 until all samples have been read.

4.2.17 Carefully **record all results** and store run file from the Qubit on a memory stick.

4.2.18 All negative controls should ideally be 'too low' to read on the Qubit machine, but MUST be < 1ng per ul. If your negative controls >1ng per ul, considerable contamination has occurred and you must redo previous steps.

15 **OPTIONAL PAUSE POINT:** PCR products can be stored in the fridge for up to a month or longer term at **-20 °C** .

Normalisation 1h 30m

16 Amplicon pools for each sample need to be combined in equimolar amounts to a total input of **5 ng** prior to barcode ligation in the next step. To do this first dilute each amplicon pool to **1 ng/µL** and then combine **2.5 µl** from each pool in a new tube with **7.5 µl** of nuclease-free water. A worked example and a normalisation template are provided below.

			1. dilute each pool to 1ng/ul in a new tube (keep pools separate)			*2. Combine normalised pools in a new tube*	
Sample id	**Pool**	**Concentration (ng/uL)**	**Volume of sample (ul)**	**Volume of water (ul)**		**Volume of dilution required (ul)**	**Volume of water (ul)**
Sample 1	1	52.0	5.0	255.0		2.50	7.5
	2	36.0	5.0	175.0		2.50	

Worked example of dilution and normalisation required to combine multiplex primer pools in equimolar amounts for the one-pot ligation reaction.

normalisation-template.xls

Native barcoding 2h 30m

17 We use the 'one-pot ligation' protocol by Josh Quick for native barcoded ligation libraries. Methods to improve barcoding efficiency are ongoing, therefore this part of the protocol may be subject to change (take note of any comments left on the protocol for new tweaks!).

One-pot native barcoding of amplicons (Ultra II AMII ligation)
by Kirstyn Brunker

PREVIEW RUN

We use the Ultra II® ligation module for barcoding *and* adaptor ligation steps but adaptor ligation may be performed with the NEBNext Quick Ligation module as per Josh Quick's original protocol https://www.protocols.io/view/one-pot-native-barcoding-of-amplicons-sg2ebye

17.1 Set up the following reaction for each sample:

Component	Volume
DNA amplicons (5ng)	**12.5 µl**
Ultra II End Prep Reaction Buffer	**1.75 µl**
Ultra II End Prep Enzyme Mix	**0.75 µl**
Total	**15 µl**

17.2 Incubate at room temperature for **00:10:00**

Incubate at **65 °C** for **00:05:00**

Incubate on ice for **00:01:00**

17.3 Add the following directly to the previous reactions:

Component	Volume
NBXX barcode	**2.5 µl**
Ultra II Ligation Master Mix	**17.5 µl**
Ligation Enhancer	**0.5 µl**
Total	**35.5 µl**

Use one native barcode from the EXP-NBD104 (1-12) or EXP-NBD114 (13-24) per sample. Use from 6 to 24 barcodes in a library, any fewer and there will be insufficient total material to achieve good yields.

17.4 Incubate at room temperature for **00:15:00**

Incubate at **70 °C** for **00:10:00**

Incubate on ice for **00:01:00**

The 70°C incubation is to inactivate the DNA ligase to prevent barcode cross-ligation when reactions are pooled in the next step.

17.5 Pool all barcoded fragments together into a new 1.5 ml Eppendorf tube and perform a SPRI bead clean-up. Elute in 45ul.

PAUSE POINT: As long as you have not yet ligated the sequencing adapter, the library can be stored at 4 °C and continue with the prep at a later point. It is better to store at 4°C, as freezing and thawing can introduce nicks or breaks in the DNA. Several days to weeks in the fridge are possible. For longer-term storage, the library can be placed at -20 °C, though unnecessary freeze-thaw cycles should be avoided for best results.

17.6 Quantify the barcoded amplicon pools using the Quantus Fluorometer using the ONE dsDNA assay.

17.7 Set up the following AMII adapter ligation reaction:

Component	Volume
Barcoded amplicon pools	**44 µl**
AMII adaptor	**5 µl**
Ultra II Ligation Master Mix	**50 µl**
Ligation Enhancer	**1 µl**
Total	**100 µl**

The input of barcoded amplicon pools will depend on the number of barcoded pools and should be between 40 ng (8 barcodes) and 120 ng (24 barcodes).

17.8 Incubate at room temperature for **00:20:00**

17.9 Add **100 µl** (1:1) of SPRI beads to the sample tube and mix gently by either flicking or pipetting.

Vortex SPRI beads thoroughly before use to ensure they are well resuspended, the solution should be a homogenous brown colour.

17.10 Pulse centrifuge to collect all liquid at the bottom of the tube.

17.11 Incubate at **37 °C** for **00:10:00** .

17.12 Place on magnetic rack and incubate for **00:02:00** or until the beads have pelleted and the supernatant is completely clear.

17.13 Carefully remove and discard the supernatant, being careful not to touch the bead pellet.

17.14 Add **200 µl** SFB and resuspend beads completely by pipette mixing.

SFB will remove excess adapter without damaging the adapter-protein complexes. Do not use 70% ethanol as in early clean-ups.

17.15 Pulse centrifuge to collect all liquid at the bottom of the tube.

17.16 Remove supernatant and discard.

17.17 Repeat steps 14-16 to perform a second SFB wash.

17.18 Pulse centrifuge and remove any residual SFB.

You do not need to allow to air dry with SFB washes.

17.19 Add **13 µl** EB and resuspend beads by pipette mixing.

17.20 Incubate at **37 °C** for **00:10:00** .

17.21 Place on magnetic rack.

17.22 Transfer final library to a new 1.5mL Eppendorf tube.

18 Quantify the final library using the Quantus Fluorometer using the ONE dsDNA assay/ Qubit Fluorometer with the high sensitivity dsDNA assay

For best results proceed directly to MinION sequencing but the final library can be now be stored in EB at 4°C for up to a week if needed.

MinION sequencing 20m

19 Prime the flowcell and load **20 ng** sequencing library onto the flowcell.

Priming and loading a MinION flowcell
by Josh Quick
PREVIEW RUN

From experience we know 20 ng is optimum loading input for short amplicons.

19.1 Thaw the following reagents at room temperature before placing on ice:

Sequencing buffer (SQB)
Loading beads (LB)
Flush buffer (FLB)
Flush tether (FLT)

19.2 Add **30 µl** FLT to the FLB tube and mix well by vortexing.

19.3 If required place a new MinION flowcell onto the MinION by flipping open the lip and pushing one end of the flowcell under the clip and pushing down gently.

19.4 Rotate the inlet port cover clockwise by 90° so that the priming port is visible.

19.5 Take a P1000 pipette and tip and set the volume to **800 µl** . Place the tip in the inlet port and holding perpendicularly to the plane of the flowell remove any air from the inlet port by turning the volume dial anti-clockwise.

Be careful not to remove so much volume that air is introduced onto the rectangular array via the outlet.

19.6 Load **800 µl** of FLB (plus FLT) into the flow cell via the inlet port, dispense slowly and smoothly trying to avoid the introduction of any air bubbles.

19.7 Wait for **00:05:00** .

19.8 Gently lift the SpotON cover to open the SpotON port.

19.9 Load another **200 µl** of FLB (plus FLT) into the flow cell via the inlet port, this will initiate a siphon at the SpotON port to allow you to load the library dilution.

19.10 In a new tube prepare the library dilution for sequencing:

Component	Volume
SQB	37.5 µl
LB	25.5 µl
Final library	12 µl
Total	75 µl

Mix LB immediately before use as they settle quickly.

Dilute library in EB if required.

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

19.12 Add the 75 µl library dilution to the flow cell via the SpotON sample port in a dropwise fashion. Ensure each drop siphons into the port before adding the next.

19.13 Gently replace the SpotON sample port cover, making sure the bung enters the SpotON port, close the inlet port and close the MinION lid.

20 Start the sequencing run using MinKNOW.

Starting a MinION sequencing run using MinKNOW
by Josh Quick

PREVIEW RUN

20.1 If required plug the MinION into the computer and wait for the MinION and flowcell to ben detected.

20.2 Choose flow cell 'FLO-MIN106' from the drop-down menu.

20.3 Then select the flowcell so a tick appears.

20.4 Click the 'New Experiment' button in the bottom left of the screen.

20.5 On the New experiment popup screen, select the running parameters for your experiment from the individual tabs:

Experiment: Name the run in the experiment field, leave the sample field blank.

Kit: Selection: Select LSK109 as there is no option for native barcoding (NBD104).

Run Options: Set the run length to 6 hours (you can stop the run once sufficient data has been collected as determined using RAMPART).

Basecalling: Leave basecalling turned but select 'fast basecalling'.

Output: The number of files that MinKNOW will write to a single folder. By default this is set to 4000 but can be reduced to make RAMPART update more frequently.

Click 'Start run'.

20.6 Monitor the progress of the run using the MinKNOW interface.