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TITLE:

Whole-Genome Sequencing for Rapid Characterization of Rabies Virus using Nanopore Technology

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KEYWORDS:

whole genome sequencing, rabies virus, genomic surveillance, nanopore sequencing, MADDOG

SUMMARY:

We present a rapid and cost-effective workflow for characterizing rabies virus (RABV) genomes using nanopore technology. The workflow is intended to support genomics-informed surveillance at a local level, providing information on circulating RABV lineages and their placement within regional phylogenies to guide rabies control measures.

ABSTRACT:

Genomic data can be used to track the transmission and geographic spread of infectious diseases. However, the sequencing capacity required for genomic surveillance remains limited in many low- and middle-income countries (LMICs,) where dog-mediated rabies and/or rabies transmitted by wildlife such as vampire bats pose major public health and economic concerns.

We present a rapid and affordable sample-to-sequence-to-interpretation workflow using nanopore technology. Protocols for sample collection and diagnosis of rabies are briefly described, followed by details of the optimized whole genome sequencing workflow, including primer design and optimization for multiplex PCR, a modified low-cost sequencing library preparation, sequencing with live and offline base calling, genetic lineage designation, and phylogenetic analysis. Implementation of the workflow is demonstrated, and critical steps are highlighted for the local deployment, such as pipeline validation, primer optimization, inclusion of negative controls, and use of publicly available data and genomic tools (GLUE, MADDOG) for classification and placement within regional and global phylogenies. The turnaround time for the workflow is 2-3 days, and the cost ranges from $25 per sample for a 96 samples run to $80 per sample for a 12 samples run. We conclude that setting up RABV genomic surveillance in LMICs is feasible and can support progress towards the global goal of zero dog-mediated human rabies deaths by 2030, as well as enhanced monitoring of wildlife rabies spread. Moreover, the platform can be adapted for other pathogens, helping to build versatile genomic capacity that contributes to epidemic and pandemic preparedness.

INTRODUCTION:

The rabies virus is a lyssavirus in the *Rhabdoviridae* family that causes a fatal neurological disease in mammals[1]. Although rabies is 100% preventable by vaccination, it remains a major public health and economic concern in endemic countries. Of the 60,000 human rabies deaths estimated to occur each year, over 95% are in Africa and Asia where dogs are the primary reservoir[2]. In contrast, dog vaccination has led to the elimination of dog-mediated rabies across Western Europe, North America, and much of Latin America. In these regions, reservoirs of rabies are now restricted to wildlife such as bats, raccoons, skunks, and wild canids[3]. Across Latin America, the common vampire bat is a problematic source of rabies due to regular spillover transmission from bats to both humans and livestock during nightly blood feeding[4]. The annual global economic impact of rabies is estimated to be 8.6 billion USD, with livestock losses accounting for 6%[5].

Sequence data from viral pathogens combined with metadata on the timing and source of infections can provide robust epidemiological insights[6]. For rabies virus (RABV), sequencing has been used to investigate the origin of outbreaks[7,8], identify host associations with wildlife or domestic dogs[8–11], and trace sources of human cases[12–14]. Outbreak investigations using phylogenetic analysis have indicated that rabies emerged in the formerly rabies-free province of Bali, Indonesia through a single introduction from the nearby endemic areas of Kalimantan or Sulawesi[15], while in the Philippines, an outbreak on Tablas island, Romblon Province was proven to be introduced from the main island of Luzon[16]. Viral genomic data have also been used to better understand pathogen transmission dynamics required for targeting control measures geographically. For example, genomic characterization of RABV illustrates geographic clustering of clades[17–19], co-circulation of lineages[20–22], human-mediated viral movement[17,23,24], and metapopulation dynamics[25,26].

Disease monitoring is one important function of genomic surveillance that has been enhanced with the global increase in sequencing capacity in response to the SARS-CoV-2 pandemic.

89 Genomic surveillance supported real-time tracking of SARS-COV-2 variants of concern[27,28] and
90 associated countermeasures[6]. Advances in accessible sequencing technology, such as nanopore
91 technology, have led to improved and more affordable protocols for rapid sequencing of both
92 human[29–32] and animal[33–35] pathogens. However, in many rabies endemic countries, there are
93 still barriers to operationalizing pathogen genomic surveillance, as shown by global disparities in
94 SARS-CoV-2 sequencing capacity[36]. Limitations in laboratory infrastructure, supply chains, and
95 technical knowledge make the establishment and routinization of genomic surveillance
96 challenging. In this paper, we demonstrate how an optimized rapid, and affordable whole
97 genome sequencing workflow can be deployed for RABV surveillance in resource-limited settings.
98
99 **PROTOCOL**
100 The study was approved by the Medical Research Coordinating Committee of the National
101 Institute for Medical Research (NIMR/HQ/R.8a/vol.IX/2788), the Ministry of Regional
102 Administration and Local Government (AB.81/288/01), and Ifakara Health Institute Institutional
103 Review Board (IHI/IRB/No:22-2014) in Tanzania; the University of Nairobi Institute of Tropical
104 and Infectious Diseases (P947/11/2019) and the Kenya Medical Research Institute (KEMRI-SERU
105 protocol No. 3268) in Kenya; and the Research Institute for Tropical Medicine (RITM),
106 Department of Health (2019-023) in the Philippines. Sequencing of samples originating from
107 Nigeria was undertaken on archived diagnostic material collected as a part of national
108 surveillance.
109
110 NOTE: Steps 1–4 are prerequisites, Steps 5–16 describe the sample-to-sequence-to-
111 interpretation workflow for RABV nanopore sequencing (**Figure 1**). For subsequent steps in the
112 protocol that needs pulse centrifugation, do 5-15 seconds at 10-15000 g.
113
114 **1. Computational environment set-up for sequencing and data analysis**
115
116 1.1. Open the Oxford Nanopore Technology (ONT) website[37] and create an account to access
117 nanopore-specific resources.
118
119 1.1.1. Log in and install ONT sequencing and basecalling software[38].
120
121 1.2. Open GitHub[39] and create an account.
122
123 1.2.1. Go to the artic-rabv[40] and MADDOG repositories[41] and follow the installation instructions.
124
125 **2. Design or update the multiplex primer scheme**
126
127 NOTE: Existing RABV schemes are available in the Artic-rabv repository[40]. When targeting a new
128 geographic area, a new scheme should be designed, or an existing scheme modified to
129 incorporate additional diversity.
130
131 2.1. Choose a genome reference set to represent the diversity in the study area - this is typically
132 a set of publicly available sequences (e.g., from NCBI GenBank) or preliminary in-house data.

133 Follow steps 2.1.1–2.1.4 to use RABV-GLUE[42], a RABV sequence data resource, to filter and
134 download NCBI sequences and associated metadata.
135
136 NOTE: Choose reference sequences with complete genomes i.e., without gaps and masked bases.
137 Choosing up to 10 sequences as a reference set for primer design is recommended. If the
138 available sequence data is incomplete or not representative of the study area, refer to the
139 advice[43–45] in **Supplemental File 1**.
140
141 2.1.1. Navigate to the **NCBI RABV Sequences by Clade** page from the **Sequence Data** drop-down
142 menu on RABV-GLUE. Click the **Rabies Virus (RABV)** link to access all available data or select a
143 particular clade of interest. Use the filter option to **Add filters** that fit the desired criteria e.g.,
144 country of origin, sequence length. Download sequences and metadata.
145
146 2.2. Generate a primer scheme for multiplex PCR following the instructions provided by Primal
147 Scheme[46]. A 400 bp scheme with a 50 bp overlap is recommended to sequence low quality
148 samples. Download and save all outputs (do not edit the file or primer names).
149
150 NOTE: The scheme will be indexed to the first sequence in the input fasta, henceforth referred
151 to as the 'index reference' (**Figure 2**). See **Supplemental File 1** for options to optimize primer
152 performance.
153
154 **3. Set up RAMPART and ARTIC bioinformatics pipeline**
155
156 3.1. Refer to **Supplemental File 2** to set up a directory structure to manage input/output files for
157 RAMPART and the ARTIC bioinformatics pipeline.
158
159 **4. Biosafety and laboratory setup**
160
161 4.1. Handle potentially rabies positive samples in Biosafety Level (BSL) 2 or 3 conditions.
162
163 4.2. Ensure laboratory staff have completed rabies pre-exposure vaccination and undergo
164 monitoring of immunity according to WHO recommendations[3].
165
166 4.3. Ensure dedicated standard operating procedures and risk assessments, following national or
167 international guidelines are in place for the laboratory.
168
169 4.4. *Required lab set-up:* Minimize contamination by maintaining physical separation between
170 pre- and post-PCR areas. In laboratories with limited space or in field lab settings use portable
171 glove boxes or makeshift lab stations to minimize contamination.
172
173 4.5. In this protocol ensure designating separate areas for:
174
175 4.5.1. Sample extraction: Set up a BSL2/3 cabinet/glove box to handle biological material and
176 perform inactivation and RNA extraction.

177
178 4.5.2. Template area: Set up a BSL1 cabinet/glove box for the addition of template (RNA/cDNA)
179 to pre-prepared reaction master mix.
180
181 4.5.3. Master mix area: Set up a designated clean area (BSL1 cabinet/glove box) for preparation
182 of reagent master mixes. No template in this area.
183
184 4.5.4. Post-PCR area: Set up a separate area for work on amplicons and sequencing library
185 preparation.
186
187 NOTE: All areas should be cleaned with a surface decontaminant and UV sterilized before and
188 after use.
189
190 **5. Field sample collection and diagnosis**
191
192 NOTE: Samples must be collected by trained and immunized personnel wearing personal
193 protective equipment and following the referenced standard procedures[47–49].
194
195 5.1. Collect sample via the foramen magnum i.e., the occipital route, as described in detail in
196 Mauti et al. 2020[50].
197
198 5.2. Diagnose rabies in the field with rapid diagnostic tests and confirm in the laboratory using
199 recommended procedures[47] such as the Direct Fluorescent Antibody Test (DFA), the Direct Rapid
200 Immunohistochemical Test (DRIT)[51,52] or the real time RT-PCR[53]
201
202 5.3. Use confirmed positive brain samples for RNA extraction or store in the freezer at -20 °C for
203 2–3 months and -80 °C for longer periods. Preserve RNA for storage and transport using a suitable
204 DNA/RNA stabilization medium.
205
206 **6. Sample preparation and RNA extraction (3 h)**
207
208 NOTE: Use a spin-column based viral RNA extraction kit suitable for the sample type.
209
210 6.1. Using a wooden applicator remove a ~3 mm cube of brain tissue (from fresh, thawed or RNA-
211 stabilized sample). 3 mm is about the height of a font 12 in print.
212
213 6.2. Label a pre-filled reinforced 2 mL tube with 1.4 mm ceramic beads or prepare a tube
214 manually by adding a quantity of beads approximate to one fill of a 200 µL PCR tube.
215
216 6.3. Disrupt the tissue in the RNA extraction kit's lysis buffer, using manufacturer's recommended
217 volume, until fully homogenized. Allow foam to settle and homogenize again if required.
218
219 NOTE: Use closed tube bead-based homogenization to limit sample exposure. If not possible, use
220 other suitable mechanical disruptors (e.g., rotor-based) or a manual micro pestle. However, these

221 may be less effective than bead beating on hard to disrupt tissue (tissue samples may harden in
222 certain storage media).
223
224 6.4. Centrifuge the lysate as per the manufacturer's instruction and use a pipette to transfer the
225 supernatant to a new microcentrifuge tube. Use only this supernatant in subsequent steps.
226
227 6.5. Follow the RNA extraction kit's spin column instructions to obtain purified RNA.
228
229 6.6. Include a negative extraction control (NEC) here and take all the way through to the
230 sequencing stage.
231
232 **7. cDNA preparation (20 min)**
233
234 7.1. In the master mix area, prepare a master mix for first strand cDNA synthesis according to the
235 number of samples and controls to be processed (with an excess volume of 10% to ensure
236 adequate reagent; **Table 1**). A no template control (NTC) should be included at this stage.
237
238 7.2. Label 0.2 ml PCR strip tubes and aliquot 5 µL of the master mix into tubes.
239
240 7.3. Take the prepared tubes to the Template area. Add 5 µL of RNA into each labelled tube,
241 including the NEC. Add 5 µL of Nuclease free water (NFW) to the NTC.
242
243 7.4. Incubate in a thermal cycler following the conditions mentioned in **Table 1**.
244
245 NOTE: Optional Pause Point: cDNA can be stored at -20°C for up to a month, if necessary, but
246 proceeding to PCR is preferred.
247
248 **8. Primer pool stock preparation (1 h)**
249
250 NOTE: This step is only necessary if making new stocks from individual primers, after which pre-
251 prepared stock solutions can be used.
252
253 8.1. Prepare a primer pool of 100 µM stock in the master mix area.
254
255 8.2. Resuspend the lyophilized primers in 1x TE or NFW at a concentration of 100 µM each. Vortex
256 thoroughly and spin down.
257
258 NOTE: In the following steps individual primers are separated into two primer pools; odd
259 numbered (named Pool A) and even numbered (named Pool B) to avoid interactions between
260 primers flanking amplicon overlaps. These pools of primers generate overlapping 400bp
261 amplicons spanning the target genome.
262
263 8.3. Arrange all odd numbered primers in a tube rack. Generate a primer pool stock by adding 5
264 µL from each primer to a 1.5 mL microcentrifuge tube labeled "primer scheme name - Pool A

265 (100 µM)".
266
267 8.4. Repeat the process for all even numbered primers and label "primer scheme name - Pool B
268 (100 µM)".
269
270 8.5. Dilute each primer pool 1:10 in molecular grade water, to generate 10 µM primer stocks.
271
272 NOTE: Make multiple aliquots of 10 µM primer dilutions and freeze them in case of degradation
273 or contamination.
274
275 **9. Multiplex PCR (5 h)**
276
277 9.1. Prepare two PCR master mixes, one for each of primer Pool A and primer Pool B in the master
278 mix area.
279
280 9.1.1. Use a final concentration of 0.015 µM per primer. Calculate the required primer pool
281 volume for the PCR reaction (**Table 2**) using the following formula:
282
283 Primer pool volume = Number of primers x Reaction volume x 0.015/ Concentration (µM) of
284 primer stock
285
286 9.2. Aliquot 10 µl each of Pool A master mix and Pool B master mix to labeled PCR strip tubes in
287 the *Template area.* For every sample, add 2.5 µl of cDNA (from step 3) to each of the
288 corresponding labeled primer pool A and B reactions. Excess cDNA can be stored at -20 °C.
289
290 9. 3. Mix by gentle flicking and pulse centrifuge.
291
292 9.4. Incubate samples with the conditions mentioned in **Table 2** on a PCR machine:
293
294 NOTE: The program does not include a specific extension step due to the long annealing time of
295 5 min (required due to the high number of primers) and the short length of the amplicons (400
296 bp) which is sufficient for the extension.
297
298 **10. PCR clean-up and quantification (3.5 h)**
299
300 10.1. Perform all work from this point on in *the post-PCR area.*
301
302 10.2. Aliquot solid-phase reversible immobilization (SPRI) beads into microcentrifuge tubes from
303 the main bottle. Store at 4 °C.
304
305 10.3. Warm a SPRI bead aliquot to room temperature (RT; ~20 °C) and thoroughly vortex until
306 beads are fully resuspended in solution.
307
308 10.4. In 1.5 mL tubes, combine primer pool A and primer pool B PCR products for each sample. If

necessary, add water to bring the volume to 25 µL.

10.5. Add 25 µL of SPRI beads to each sample (1:1 beads:sample ratio). Mix by pipetting up and down or gently tap tube.

10.6. Incubate at RT for 10 min, occasionally inverting or flicking tubes.

10.7. Place on a magnetic rack until the beads and solution have fully separated. Remove and discard supernatant, taking care not to disturb the bead pellet.

10.8. Wash twice with 80% ethanol (warmed to RT).

10.8.1. Add 200 µL of ethanol to the pellet. Wait for 30 s to ensure the beads are washed properly.

10.8.2. Carefully remove as much ethanol as possible using a 10 µL tip.

10.8.3. Repeat steps 10.8.1–10.8.2 to wash pellet for a second time.

10.9. Remove all traces of ethanol. Air dry until trace ethanol has evaporated (~1 min), when this happens the pellet should go from shiny to matt. Take care not to overdry (if pellet is cracking it is too dry) as this will affect DNA recovery.

10.10. Resuspend the beads in 15 µL of NFW and incubate at RT (off magnetic rack) for 10 min.

10.11. Return to the magnetic rack and transfer the supernatant (cleaned product) to a fresh 1.5 mL tube.

10.12. Prepare a 1:10 dilution of each sample (2 µL of product +18 µL of NFW).

NOTE: Be very careful at this stage to avoid cross-contamination. Only have one amplicon tube open at a time. Aliquot 18 µL of water into the tubes first (in clean *master mix area*).

10.13. Measure the DNA concentration of each diluted sample using a highly sensitive and specific fluorometer as described in protocols.io[54,55].

11. Normalization (30 min)

11.1. Use the normalization template (**Supplemental File 3**) and DNA concentration (ng/µL) of each sample to calculate the volume of diluted (or neat) sample required for 200 fmol of each sample in a total volume of 5 µL.

11.2. Label new PCR tubes and add computed volumes of NFW and sample to obtain normalized DNA.

353 11.3. Use the computed volume for undiluted (neat) samples if over 5 µL of the diluted sample is
354 required to obtain 200 fmol.
355
356 NOTE: Optional Pause Point: At this point the cleaned-up PCR product can be stored at 4 °C for
357 up to a week or placed at -20°C for longer-term storage if needed
358
359 **12. End prep and barcoding (1.5 h)**
360
361 NOTE: The next steps assume use of specific reagents from nanopore-specific barcoding and
362 ligation sequencing kits, please refer to the Materials list in (**Supplemental File 4**) for details. The
363 protocol is transferable across different chemistry versions, but user should take care to use
364 compatible kits according to the manufacturer's information.
365
366 12.1. End repair and dA-tailing
367
368 12.1.1. Set up the end-prep reaction for each sample mentioned in **Table 3**. Prepare a master mix
369 according to the number of samples (plus 10% excess). Take care when pipetting as reagents are
370 viscous.
371
372 12.1.2. Add 5 µl of master mix into each tube of normalized DNA (5 µl). Total reaction mix should
373 be 10 µl. Change tips each time and only have one tube open at a time.
374
375 12.1.3. Incubate in a thermal cycler under the conditions mentioned in **Table 3**.
376
377 12.2. Barcoding
378
379 12.2.1. Aliquot the barcodes from the barcoding kit to PCR strip tubes at 1.25 µL/tube, record
380 barcode assigned to each sample.
381
382 12.2.2. Add 0.75 µL of the end prepped sample to its assigned barcode aliquot.
383
384 12.2.3. Set up the ligation reaction for each sample as follows. Prepare a master mix according
385 to the number of samples (plus 10% excess) (**Table 4**).
386
387 12.2.4. Add 8 µl of ligation master mix to end-prepped sample + barcodes, giving a total reaction
388 of 10 µl.
389
390 12.2.5. Incubate in a thermal cycler using the conditions mentioned in **Table 4**.
391
392 12.3. SPRI bead clean-up and DNA quantification
393
394 12.3.1. Thaw Short Fragment Buffer (SFB) at RT, mix by vortexing, pulse centrifuge, and place on
395 ice.
396

12.3.2. Pool all barcoded samples together in a 1.5 mL lobind microcentrifuge tube. So as not to make the clean-up volume too large to use: 12–24 samples (10 µL), up to 48 samples (5 µL), up to 96 samples (2.5 µL) from each native barcoding reaction.

12.3.3. Add 0.4x volume of SPRI beads to barcoded pool. Mix gently (flicking or pipetting) and incubate at RT for 5 min.

12.3.4. Place the samples on magnet until beads have pelleted and supernatant is completely clear (~2 min). Remove and discard the supernatant. Take care not to disturb the beads.

12.3.5. Wash twice with 250 µL of SFB.

12.3.6. Remove the tube from magnet and resuspend the pellet in 250 µL of SFB. Incubate for 30 s, pulse centrifuge and return to magnet. Remove supernatant and discard.

12.3.7. Repeat step 12.3.6 to perform a second SFB wash.

12.3.8. Pulse centrifuge and remove any residual SFB.

12.3.9. Add 200 µL of 80% (RT) ethanol to bathe the pellet. Remove and discard ethanol being careful not to disturb the bead pellet. Air dry for 30 s or until the pellet has lost its shine.

12.3.10. Resuspend in 22 µL of NFW at RT for 10 min.

12.3.11. Place on magnet, leave to settle for ~2 min, then carefully remove solution and transfer to a clean 1.5 mL microcentrifuge tube.

12.3.12. Use 1 µL to obtain DNA concentration as described previously (section 10.13).

NOTE: Optional Pause Point: At this point the library can be stored at 4 °C for up to a week or -20 °C for longer-term storage, but it is preferable to continue with adapter ligation and sequencing.

13. Sequencing (48 h maximum)

13.1. Prepare computer (refer also to **Prerequisites** sections 1–4)

13.1.1. Check there is enough space to store new data (min 150 GB); that data from old runs is backed up/moved to server before deleting and that the latest version of MinKNOW is installed.

13.2. Remove the stored flow cell from the fridge and allow to reach RT.

13.3. Adapter ligation (1 h)

441 13.3.1. Pulse centrifuge the adapter mix and ligase and place on ice
442
443 13.3.2. Thaw elution buffer (EB), SFB and ligation buffer at RT. Mix by vortexing, pulse centrifuge,
444 place on ice
445
446 13.3.3. Prepare the adapter ligation master mix below, combining reagents in specified order in
447 a low bind tube:
448
449 NOTE: Alternatives for adapter ligation master mix reagents (**Table 5**) can be used depending on
450 availability at the lab. See **Supplemental File 3** and **Supplemental File 4** for list of alternatives.
451 Use computation in Supplemental File 3 worksheet to get the volume of DNA library equivalent
452 to 200 fmol. If less than 20 µL is computed, add NFW to make up to 20 µL.
453
454 13.3.4. Mix by gentle flicking and pulse centrifuge. Incubate at RT for 20 min.
455
456 NOTE: During incubation start preparing the flow cell (section 13.5).
457
458 13.4. Clean up using SPRI beads (do not use ethanol as in earlier cleanups)
459
460 13.4.1. Add 0.4x volume of SPRI beads (RT) to the samples. Incubate at RT for 10 min, gently flick
461 and invert intermittently to aid mixing.
462
463 13.4.2. Place on magnet until beads and solution have fully separated (~5 min). Remove and
464 discard the supernatant; take care not to disturb the bead pellet.
465
466 13.4.3. Wash twice with 125 µL of SFB.
467
468 13.4.4. Resuspend the pellet completely with 125 µL of SFB by pipette mixing. Leave to incubate
469 for 30 s.
470
471 13.4.5. Pulse centrifuge to collect liquid at tube base and place on magnet. Remove the
472 supernatant and discard.
473
474 13.4.6. Repeat step 13.4.4 - 13.4.5 to wash pellet for a second time.
475
476 13.4.7. Pulse centrifuge and remove the excess SFB.
477
478 13.4.8. Resuspend in 15 µL of EB and incubate for 10 min at RT.
479
480 13.4.9. Return to magnet for ~2 min and then carefully transfer solution to a clean 1.5 mL
481 microcentrifuge tube.
482
483 13.4.10. Quantify 1 µL of the eluted library as described previously on step 10.13.
484

485 NOTE: For best results proceed directly to MinION sequencing but the final library can be stored
486 in EB at 4 °C for up to a week if needed.
487
488 13.5. Run a flow cell quality check.
489
490 13.5.1. Connect the sequencing device to laptop and open the sequencing software.
491
492 13.5.2. Select flow cell type and click **Check Flow Cell** then **Start Test**
493
494 13.5.3. Once complete, the total number of active (i.e., viable) pores will be displayed. A new
495 flow cell should have >800 active pores, if it does not contact manufacturer for a replacement.
496
497 13.6. Priming and loading the flow cell (20min)
498
499 13.6.1. Thaw the following reagents at room temp then place on ice: sequencing buffer, flush
500 tether, flush buffer, loading beads
501
502 13.6.2. Vortex the sequencing buffer and flush buffer, pulse centrifuge, and place on ice
503
504 13.6.3. Pulse centrifuge flush tether and mix by pipetting, place on ice.
505
506 13.6.4. Prepare the flow cell priming mix by adding 30 µL of flush tether directly to the tube of
507 flush buffer from a flow cell priming kit and mix by pipetting.
508
509 13.6.5. Mix the loading beads by pipetting immediately prior to use as they settle quickly.
510
511 13.6.6. In a fresh tube prepare the final library dilution for sequencing as mentioned in **Table 5**.
512
513 NOTE: Use computation in Supplemental File 3 worksheet to get the volume of DNA
514 library equivalent to 50 fmol. If less than 12 ul is computed, add elution buffer to make
515 up to 12 ul.
516
517 13.6.7. Flip back the sequencing device lid and slide the priming port cover clockwise so that
518 priming port is visible (**Figure 4**)
519
520 13.6.8. Remove air bubbles carefully by setting a P1000 to 200 µL, insert the tip into the priming
521 port and turn the wheel until you can see a small volume entering the pipette tip (max turn to
522 230 µL)
523
524 13.6.9. Load 800 µL of flow cell priming mix into the flow cell via the priming port taking care to
525 avoid bubbles.
526
527 13.6.10. Leave for 5 min.
528

529 13.6.11. Lift the sample port cover gently and load 200 µL of priming mix into flow cell via Priming
530 port using a P1000 pipette
531
532 13.6.12. Pipette mix library mix prior to loading ensuring loading beads in the master mix are
533 resuspended before loading.
534
535 13.6.13. Load 75 µL of library mix to flow cell via the sample port in a dropwise fashion. Ensure
536 that each drip flows into the port before adding the next.
537
538 13.6.14. Replace the sample port cover gently making sure bung enters sample port.
539
540 13.6.15. Close the priming port and replace the sequencing device lid.
541
542 13.7. Sequencing run (48 h maximum)
543
544 13.7.1. Connect the sequencing device to the laptop and open sequencing software.
545
546 13.7.2. Click start and then click **Start Sequencing**.
547
548 13.7.3. Click **New Experiment** and follow the sequencing software GUI workflow to set up the
549 parameters for the run.
550
551 13.7.4. Type in the Experiment name, Sample ID (e.g., rabv_run1) and choose the **Flow Cell Type**
552 from the drop-down menu.
553
554 13.7.5. Continue to kit selection and choose the relevant ligation sequencing kit and native
555 barcoding kit(s) used.
556
557 13.7.6. Continue to **Run** options. Keep defaults unless you want the run to stop automatically
558 after a certain number of hours (runs can be stopped manually at any time).
559
560 13.7.7. Continue to **Basecalling**. Choose to turn **Basecalling On** or **Off** according to the computing
561 resources (see computer setup). Choose Edit Options under barcoding and ensure Barcode Both
562 Ends is turned on. Save and continue to Output section.
563
564 13.7.8. Accept defaults and continue to final review, check settings and record details in
565 worksheet (**Supplemental File 3**). Click **Start**.
566
567 NOTE: If flow cell is being reused adjust the starting voltage (in the advanced section of the run
568 options) as indicated by the scheme in **Supplemental File 3**.
569
570 13.7.9. Record the initial active channels - if this is significantly lower than the QC check then
571 restart the sequencing software, if still lower reboot the computer.
572

573 13.7.10. Record the initial channels in strand vs single pore to give an approximate pore
574 occupancy. This number will fluctuate so give an approximation.
575
576 13.7.11. Monitor the run as it progresses.
577
578 **14. Live and offline basecalling**
579
580 NOTE: These instructions assume the pre-existing directory structure provided in the artic-rabv
581 repository, and that the Prerequisites Step 1 and 3 of the Protocol have been followed.
582
583 14.1. In the artic-rabv/analysis folder, navigate to the relevant project directory and create a new
584 directory for the run using the Sample ID provided to MinKNOW as the run_name.
585 **cd path/artic-rabv/analysis/project_name**
586 **mkdir -p run_name/**
587
588 14.2. Live basecalling
589
590 ***NOTE:*** To perform Nanopore basecalling in real-time, laptops require a NVIDIA CUDA-compatible
591 GPU. Ensure instructions for GPU basecalling setup have been performed using the guppy
592 protocol[56]
593
594 14.2.1. During run setup, turn live basecalling on.
595
596 14.2.2. Use RAMPART to monitor the sequencing coverage in real time as per instruction below.
597
598 14.2.3. In the computer's terminal, activate the artic-rabv conda environment:
599 **conda activate artic-rabv**
600
601 14.2.4. Create a new directory for the rampart output inside the run_name directory and
602 navigate into it:
603 **cd /path/artic-rabv/analysis/project_name/run_name**
604 **mkdir rampart_output**
605 **cd rampart_output**
606
607 14.2.5. Create a **barcodes.csv** file to pair barcodes and sample names. It should have one line per
608 barcode and only specify barcodes that are present in your library, with the headings "barcode"
609 and "sample". Follow the example in the artic-rabv directory:
610 **artic-**
611 **rabv/analysis/example_project/example_run/rampart_output/**
612 **barcodes.csv**
613
614 14.2.6. Start RAMPART by providing the relevant protocol folder and path to the fastq_pass folder
615 in the MinKNOW output for the run:
616 **rampart --protocol /path/artic-**

rabv/rampart/scheme_name_V1_protocol –basecalledPath <insertpathTo Fastqpassfolder>

10.2.7. Open a browser window and navigate to localhost:3000 in the URL box. Wait for sufficient data to be basecalled before results appear on the screen.

14.3. Offline basecalling (performed post-run)

14.3.1. If live basecalling was not set, the output from MinKNOW will be raw signal data (fast5 files). You will not be able to use RAMPART during the run. Fast5 files can be converted to basecalled data (fastq files) post-run using Guppy (see setup in Prerequisites 1.1.1.). You can run RAMPART post-hoc on basecalled data.

14.3.2. Run the guppy basecaller:

guppy_basecaller -c dna_r9.4.1_450bps_fast.cfg -i /path/to/reads/fast5_* -s /path/artic-rabv/analysis/project_name/run_name -x auto -r

-c is the config file to specify the basecalling model, -i is the input path, -s is the save path, -x specifies basecalling by GPU device (exclude if using CPU version of Guppy), -r specifies to search input files recursively. fast5_* will search both the fast5_pass and fast5_fail folders.

NOTE: the config file (.cfg) can be changed to a High Accuracy basecaller by replacing _fast with _hac. This will take significantly longer.

15. Washing flow cells

15.1. Flow cells can be washed and reused to sequence new libraries if pores are still viable. See instructions for washing at ONT flow cell wash protocol[57].

16. Analysis and Interpretation

16.1. Consensus sequence generation with ARTIC bioinformatics pipeline.

16.1.1. Follow the instructions detailed in the artic-rabv GitHub repository[40] in the rabv_protocols folder to generate consensus sequences from raw fast5 or basecalled fastq files.

NOTE*:* Refer to Artic pipeline - Core pipeline[58] for further guidance.

16.2. *Optional:* Analyze the average read depth per amplicon.

16.2.1. Adapt the scripts available from the artic-rabv repository, referring to Supplemental File 1. Briefly, depth statistics are generated using SAMtools[59] and coverage per nucleotide plotted in R.

16.3. Phylogenetic analysis using GLUE.

16.3.1. From RABV_GLUE[42] select **Analysis** > **Genotyping and Interpretation** tab, and **Add Files**, selecting your fasta file of consensus sequences.

16.3.2. Click **Submit** and wait. Once analyses are complete, the **Show Analysis** button will be available to click, showing clade and subclade assignments; coverage per gene; variation from reference sequences; closest relative.

16.3.3. Relevant contextual sequences can also be identified in the **Sequence Data** > **NCBI Sequences by Clade** section.

16.3.4. Select the clade identified or click **Rabies Virus (RABV)** to see all available sequences.

16.3.5. Filter for relevant sequences (e.g., country of origin)

16.3.6. Download these sequences and corresponding metadata for analysis and comparison.

16.4. Lineage assignment using MADDOG[41].

16.4.1. Create a folder within your local MADDOG repository (previously created at the Prerequisites section), called the run name.

16.4.2. Inside the folder, add the fasta file containing your consensus sequences.

16.4.3. Add a metadata file to the folder. This file must be a csv with 4 columns called 'ID', 'country', 'year' and 'assignment', detailing the sequence IDs, the country of sampling, and year of sample collection, while the 'assignment' column should be blank.

NOTE: The ID in the metadata file must exactly match the IDs in the fasta file.

16.4.4. In the command line interface, activate the conda environment: **conda activate MADDOG.**

16.4.5. In the command line interface, navigate to the MADDOG repository folder.

16.4.6. Initially, lineage assignment is undertaken on sequences to check for any potential abnormalities, and to identify if running the longer lineage designation step would be appropriate: **sh assignment.sh**

16.4.7. When prompted, enter the name of the folder within the MADDOG repository folder that contains your fasta file.

705 16.4.8. When lineage assignment is complete, check the output file in your folder. If the output
706 is as expected, and there are multiple sequences assigning to the same lineage, lineage
707 designation should then be run.
708
709 16.4.9. If running lineage designation, delete the assignment output file just created.
710
711 16.4.10. In the terminal, inside the MADDOG repository folder, run the command: **sh**
712 **designation.sh**
713
714 16.4.11. When prompted, enter the folder name within the MADDOG repository folder
715 containing your fasta file and metadata. This will output lineage information about each
716 sequence, a phylogeny of the new and relevant previous sequences (from 16.3.6), hierarchical
717 information about the lineages, and details of potentially emerging lineages and areas of
718 undersampling.
719
720 16.4.12. Full details of the protocol, usage, and outputs in Campbell et al. 2022[60].
721
722 **REPRESENTATIVE RESULTS:**
723 The sample-to-sequence-to-interpretation workflow for RABV described in this protocol has
724 been used successfully in different laboratory conditions in endemic countries, such as Tanzania,
725 Kenya, Nigeria, and Philippines (**Figure 3**). The protocol was used on different sample types and
726 conditions (**Table 6**); fresh and frozen brain tissue, cDNA and RNA extracts from brain tissue
727 transported under cold chain for extended periods, and FTA cards with brain tissue smears.
728
729 Live basecalling using RAMPART (**Figure 5**) shows the almost real time generation of reads and
730 the percent coverage per sample. This is particularly useful in deciding when to stop the run and
731 save the flow cell for reuse. Variation in run time was observed, with some finished in two hours,
732 while others could take more than 12 hours for adequate depth of coverage (x100) to be reached.
733 We can also view regions with poor amplification e.g. **Figure 6** shows a snapshot of one
734 sequencing run where coverage profiles show some amplicons with very low amplification,
735 indicating potentially problematic primers. By investigating these poorly amplifying regions more
736 thoroughly we have been able to identify primer mismatches that will enable us to redesign and
737 improve individual primers. Some primer schemes have shown more mismatches than others.
738 This is observed in the East Africa primer scheme compared to the Philippines, in line with the
739 targeted diversity, as the East Africa scheme aims to capture a much broader diversity.
740
741 RABV-GLUE[42], a general purpose resource for RABV genome data management and MADDOG[60],
742 a lineage classification and nomenclature system were used to compile and interpret resulting
743 RABV sequences. **Table 7** shows the major and minor clades circulating in each country assigned
744 using RABV GLUE and also shown is a higher resolution classification of local lineages following
745 the MADDOG assignment.
746
747 **FIGURE AND TABLE LEGENDS:**
748 **Figure 1: Sample-to-sequence-to-interpretation workflow for RABV.** Summarized steps are

749 shown for (**A**) sample preparation, (**B**) PCR and library preparation and (**C**) sequencing and
750 bioinformatics up to analysis and interpretation.
751
752 **Figure 2: Primer scheme schematic.** Shows annealing positions along the 'Index reference
753 genome' (dark purple) for pairs of forward and reverse primers (half arrows) which are assigned
754 in two separate pools: **A** (red) and **B** (green). Primer pairs generate 400 bp overlapping amplicons
755 (blue) which are numbered sequentially along the index reference genome in the format
756 'scheme_name_X_DIRECTION' where 'X' is a number referring to the amplicon generated by the
757 primer, and 'DIRECTION' is either 'LEFT' or 'RIGHT' describing the forward or reverse respectively.
758 Odd or even value of 'X' determines the Pool A/B.
759
760 **Figure 3. Map showing location where RABV sequencing was conducted using the optimized**
761 **workflow in 2021 and 2022.** Bubble size and color correspond to the number of sequences per
762 location where darker/smaller is fewer and lighter/larger is more.
763
764 **Figure 4. Nanopore flow cell[48].** Blue labels illustrate the different parts of the flow cell, including
765 the priming port cover which covers the priming port where the priming solution is added, the
766 SpotON sample port cover covering the sample port where the sample is added in a dropwise
767 fashion, the waste ports 1 and 2, and the flow cell ID.
768
769 **Figure 5. Screenshot of RAMPART visualization in web browser.** Barcode names are replaced by
770 sample names according to bioinformatic setup. Top three panels show summary plots for the
771 whole run: depth of coverage of mapped reads for each barcode per nucleotide position on the
772 index reference genome (top left, colored by barcode) summed mapped reads from all barcodes
773 over time (top middle), mapped reads per barcode (top right, colored by barcode). Lower panels
774 show rows of plots per barcoded. From left to right: the depth of coverage of mapped reads per
775 nucleotide position on the index reference genome (left), length distribution of mapped reads
776 (middle), proportion of nucleotide positions on the index reference genome which have obtained
777 10x, 100x and 1000x coverage of mapped reads over time (right).
778
779 **Figure 6. Example sequencing depth of coverage profile for a sample from the Philippines.**
780 Depth of coverage by mapped reads (y axis) is plotted per nucleotide position on the index
781 reference genome (x axis). The approximate position of amplicons (1-41) is labeled at the top of
782 the plot. Spikes in the depth of coverage between amplicons indicate areas of amplicon overlap.
783 Amplicons with a low depth of coverage (such as 21 and 41) indicate problematic regions of the
784 genome where primers may require optimization.
785
786 **Table 1: Master mix and thermal cycler conditions for cDNA preparation.**
787
788 **Table 2: Master mix and thermal cycler conditions for multiplex PCR**
789
790 **Table 3: Master mix and thermal cycler conditions for end prep reaction**
791
792 **Table 4: Master mix and thermal cycler conditions for barcoding.**

793
794 **Table 5: Adapter ligation master mix and final library dilution for sequencing.**
795
796 **Table 6. Number of rabies virus whole genome sequences generated and the type of samples**
797 **used in different countries using the sample-to-sequence-to-interpretation workflow.**
798
799 **Table 7. Major and minor clade assignments from RABV-GLUE and lineage assignments from**
800 **MADDOG for sequences generated using the workflow.**
801
802 **DISCUSSION:**
803 An accessible RABV nanopore-based whole genome sequencing workflow was developed by
804 Brunker et al. (2020)[61] using resources from the ARTIC network.[46] Here we present an updated
805 workflow, with complete sample-to-sequence-to-interpretation steps. The workflow details the
806 preparation of brain tissue samples for whole genome sequencing, presents a bioinformatics
807 pipeline to process reads and generate consensus sequences, and highlights two rabies-specific
808 tools to automate lineage assignment and determine phylogenetic context. The updated
809 workflow also provides comprehensive instructions for the setup of appropriate computational
810 and laboratory workspaces, with considerations for implementation in different contexts
811 (including low resource settings). We have demonstrated the successful implementation of the
812 workflow in both academic and research institute settings in four RABV endemic LMICs with no
813 or limited genomic surveillance capacity. The workflow has proven resilient to application across
814 diverse settings, and comprehensible by users with varying expertise.
815
816 This workflow for rabies virus sequencing is the most comprehensive publicly available protocol
817 (covering sample-sequence-interpretation steps) and specifically adapted to reduce both startup
818 and running costs. The time and cost required for library preparation and sequencing with
819 Nanopore is greatly reduced relative to other platforms such as Illumina[61] and continual
820 technology developments are improving sequence quality and accuracy to be comparable with
821 Illumina[62].
822
823 This protocol is designed to be resilient in diverse low resource contexts. By referring to the
824 troubleshooting and modifications guidance provided alongside the core protocol, users are
825 supported to adapt the workflow to their needs. The addition of user-friendly bioinformatic tools
826 to the workflow constitutes a major development to the original protocol, providing rapid and
827 standardized methods that can be applied by users with minimal prior bioinformatics experience
828 to interpret sequence data in local contexts. The capacity to do this in situ is often limited by the
829 need to have specific programming and phylogenetic skills, which require an intensive and long-
830 term skills training investment. While this skillset is important to thoroughly interpret sequence
831 data, basic and accessible interpretation tools are equally desirable in order to capacitate local
832 "sequencing champions", whose core expertise may be wet lab based, enabling them to interpret
833 and take ownership over their data.
834
835 As the protocol has been undertaken for a number of years in several countries, we now provide
836 guidance on how to optimize multiplex primer schemes to improve coverage and deal with

837 accumulated diversity. Efforts have also been made to help users improve cost-effectiveness or
838 to allow for ease of procurement in a given region, which is typically a challenge for the
839 sustainability of molecular approaches[63]. For example, in Africa (Tanzania, Kenya and Nigeria),
840 we opted for Blunt/TA Ligase Master Mix at the adapter ligation step, which was more readily
841 available from local suppliers and a cheaper alternative to other ligation reagents.
842
843 From experience, there are several ways of reducing the cost per sample and per run. Reducing
844 the number of samples per run (e.g., from 24 down to 12 samples) can extend the life of flow
845 cells over multiple runs. Whereas increasing the number of samples per run will maximize time
846 and reagents. In our hands, we were able to wash and reuse flow cells for one in every three
847 sequencing runs, enabling an additional 55 more samples to be sequenced. Washing the flow
848 cell immediately after use or if not possible, removing the waste fluid from the waste channel
849 after every run, seemed to preserve the number of pores available for a second run. Taking into
850 consideration the initial number of pores available in a flow cell, one run can also be optimized
851 to plan how many samples to run in a particular flow cell.
852
853 Though the workflow aims to be as comprehensive as possible with addition of detailed guidance
854 and signposted resources, the procedure is still complex and can be daunting for a new user. The
855 user is encouraged to seek in-person training and support, ideally locally, or alternatively,
856 through external collaborators. In the Philippines for example, a project on capacity building
857 within regional laboratories for SARS-CoV-2 genomic surveillance using ONT has developed core
858 competencies among health care diagnosticians that are readily transferrable to RABV
859 sequencing. Important steps such as SPRI bead clean-ups can be difficult to master without
860 hands-on training and ineffective clean-ups can damage the flow cell and compromise the run.
861 Sample contamination is always a major concern when amplicons are being processed in the lab
862 and can be difficult to eliminate. In particular, cross-contamination between samples is extremely
863 difficult to detect during post-run bioinformatics. Good laboratory technique and practices such
864 as maintaining clean work surfaces, separating pre- and post-PCR areas, and incorporating
865 negative controls are imperative to ensure quality control. The fast pace of nanopore sequencing
866 developments is both an advantage and disadvantage for routine RABV genomic surveillance.
867 Continuing improvements to nanopore's accuracy, accessibility and protocol repertoire widen
868 and improve the scope for its application. However, the same developments make it challenging
869 to maintain standard operating procedures and bioinformatic pipelines. In this protocol, we
870 provide a document assisting transition from older to current nanopore library preparation kits
871 (**Supplemental File 4**).
872
873 A common roadblock to sequencing in LMICs is accessibility, including not only cost but the ability
874 to procure consumables in a timely manner (particularly sequencing reagents, which are
875 relatively new to procurement teams and suppliers), computational resources and simply having
876 access to stable power and internet. Using portable nanopore sequencing technology as the
877 foundation of this workflow helps with many of these accessibility issues and we have
878 demonstrated the use of our protocol across a range of settings, conducting the full protocol and
879 analysis in-country. Admittedly, procuring equipment and sequencing consumables in a timely
880 manner remains a challenge and, in many instances, we were forced to carry or ship reagents

881 from the UK. However, in some areas we were able to rely entirely on local supply routes for
882 reagents, benefiting from investment in SARS-CoV-2 sequencing (e.g., Philippines) that has
883 streamlined procurement processes and begun to normalize the application of pathogen
884 genomics.
885
886 The need for a stable internet connection is minimized by one-time-only installs e.g., GitHub
887 repositories, software download, and MinION sequencing itself only requires internet access to
888 start the run (not throughout) or can be performed completely offline with agreement from the
889 company. If mobile data is available, a phone can be used as a hotspot to the laptop to begin the
890 sequencing run, before disconnecting for the run duration. When routinely processing samples,
891 data storage requirements can grow rapidly and ideally data would be stored on a server.
892 Otherwise, SSD hard drives are relatively cheap to source.
893
894 While we recognize that there are still barriers to genomic surveillance in LMICs, increasing
895 investment in building genomics accessibility and expertise (e.g. Africa Pathogen Genomics
896 Initiative (Africa PGI))[64] suggests that this situation will improve. Genomic surveillance is critical
897 for pandemic preparedness[6], and capacity can be established through routinizing genomic
898 surveillance of endemic pathogens such as RABV. Global disparities in sequencing capacities
899 highlighted during the SARS-CoV-2 pandemic should be a driver of catalytic change to address
900 these structural inequities.
901
902 This sample-to-sequence-to-interpretation workflow for RABV, including accessible
903 bioinformatics tools, has potential to be used to guide control measures targeting the goal of
904 zero human deaths from dog-mediated rabies by 2030 and ultimately for the elimination of
905 rabies virus variants. Combined with relevant metadata, genomic data generated from this
906 protocol facilitates rapid RABV characterization during outbreak investigations and in
907 identification of circulating lineages in a country or region[60,61]. We illustrate our pipeline using
908 mostly examples from dog-mediated rabies; however the workflow is directly applicable to
909 wildlife rabies. This transferability and low cost minimizes the challenges to making routine
910 sequencing easily available not only for rabies but also for other pathogens[46,65,66] to improve
911 disease management and control.
912
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922
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924 The authors have nothing to disclose.

925

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