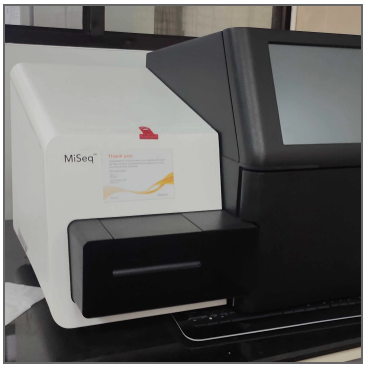

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Protocol status: Working
We use this protocol and it's working

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Rabies Virus Sequencing using Illumina- MiSeq

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ABSTRACT

This Rabies whole genome sequencing protocol has been derived and modified from the Illumina COVIDSeq RUO sequencing pipeline. The protocol has been modified and optimised for sequencing Rabies virus (RABV). The methodology uses RABV-specific primers that have been designed in-house. In brief, the RNA is extracted from samples and converted to cDNA. RABV sequencing library is generated and sequenced using MiSeq.

GUIDELINES

Ensure all Biosafety guidelines are followed as per the working policy.

Please ensure you keep a log of sample processing and use a template to locate sample wells.

The protocol is based on sequencing a total of 96 samples, which includes a known positive and negative control. At least one negative control is mandatory to assess possible contamination. Positive control with a known sequence is mandatory to estimate the quality of the run.

The complete library preparation process takes approximately two days at our laboratory. It is advisable to use "Safe Stops" as needed.

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PROTOCOL integer ID: 93845

Keywords: Sequencing, Rabies Virus, Lyssavirus Rabies, Illumina MiSeq, NGS

MATERIALS

The following reagents available from Illumina have been used in this protocol.

EPH3 (Elution Prime Fragment 3HC Mix)
FSM (First Strand Mix)
RVT (Reverse Transcriptase)
IPM (Illumina PCR Mix)
EBLTS (Enrichment BLT)
TB1 (Tagmentation Buffer 1)
Nuclease-free water (NFW)
ST2 (Stop Tagment Buffer 2)
TWB (Tagmentation Wash Buffer)
EPM (Enhanced PCR Mix)
Index adapters (Illumina-PCR Indexes)
ITB (Illumina Tune Beads)
Ethanol
RSB (Resuspension Buffer)

SAFETY WARNINGS

Use appropriate PPE as needed.

BEFORE START INSTRUCTIONS

All the processes should be performed in Biosafety cabinets. Ensure you have separate Biosafety cabinets for RNA extraction, Reagent preparation, and Template addition.

Samples and Extraction

27m 30s

1 **Samples:**
The following samples (human or animal sources) may be used for viral RNA extraction for Rabies lyssavirus.
1. Brain Tissue or Nuchal skin: Homogenise the tissue by crushing a small piece in a sterile environment. Transfer the contents to a vial and vortex and spin down the sample. Retrieve the supernatant.
2. Saliva

2 **RNA Extraction:**

The RNA is extracted using theQIAmp Viral RNA Mini Kit, as per the procedure outlined in the QIAamp Viral RNA Mini Handbook (https://www.qiagen.com/us/resources/download.aspx?id=c80685c0-4103-49ea-aa72-8989420e3018&lang=en).

2.1 Add 140 µL of the supernatant or saliva sample to the Buffer AVL–carrier RNA in the microcentrifuge tube. Mix by pulse-vortexing for 00:00:15. 15s

2.2 Incubate at Room temperature for 00:20:00. 20m

2.3 Briefly centrifuge the tube to remove drops from the inside of the lid.

2.4 Add 560 µL ethanol (96–100%) to the sample, and mix by pulse-vortexing for 00:00:1[illegible]. 15s
. After mixing, briefly centrifuge the tube to remove drops from inside the lid.

2.5 Carefully apply 630 µL of the solution from step 2.4 to the QIAamp Mini column (in a 2 mL collection tube) without wetting the rim. Close the cap, and centrifuge at 8000 rpm for 00:01:00. Place the QIAamp Mini column into a clean 2 mL collection tube, and discard the tube containing the filtrate. 1m

2.6 Carefully open the QIAamp Mini column, and repeat the step.

> **Note**
>
> If the original sample volume exceeds 140 µL, repeat this step until the lysate has been loaded onto the spin column.

2.7 Carefully open the QIAamp Mini column, and add 500 µL Buffer AW1. Close the cap, and centrifuge at 8000 rpm for 00:01:00. Place the QIAamp Mini column in a clean 2 mL collection tube (provided), and discard the tube containing the filtrate. 1m

2.8 Carefully open the QIAamp Mini column, and add 500 µL Buffer AW2. Close the cap and centrifuge at full speed 14.000 rpm for 00:03:00. 3m

2.9 Place the QIAamp Mini column in a clean 1.5 mL microcentrifuge tube. Discard the old collectio 1m tube containing the filtrate. Carefully open the QIAamp Mini column and add 60 µL of Buffer AVE. Close the cap and incubate at Room temperature for 00:01:00.

> **Note**
>
> If a very low viral load is expected, such as in a salivary sample, it is advised to elute in approximately 20 µL.

2.10 Centrifuge at 8000 rpm for 00:01:00. The eluted RNA can be used immediately or stored at ≤ -80 °C . 1m

> **Note**
>
> It is advised to check the quality of extracted RNA using Nanodrop and run an RABV RT-PCR to determine the Ct value. Sequencing does not work well if extraction quality is low or the Ct is > 30.

cDNA Conversion 25m

3 The extracted RNA is annealed using random hexamers to prepare for cDNA synthesis during this process. 5m

Thaw the EPH3 (Elution Prime Fragment 3HC Mix) at Room temperature.

Note

Use nuclease-free water as a Negative Control in one or more wells depending on the user's requirement.

Label a new PCR plate as **CDNA**.

Add 8.5 µl EPH3 to each well.
Add 8.5 µl eluted sample to each well.
Seal and shake at 1600 rpm for 00:01:00.
Centrifuge at 1000 rpm for 00:01:00.

Set up a PCR (RABV Anneal program) as follows:

65 °C for 00:03:00
Hold at 4 °C

4 This step reverse transcribes the RNA fragments primed with random hexamers into first-strand cDNA us 22m reverse transcriptase.

Thaw the FSM (First Strand Mix) and RVT (Reverse Transcriptase) reagents in Room temperature

For 96 samples, prepare a master mix in a 1.7 ml tube as follows.

FSM- 720 µL
RVT- 80 µL

Add 8 µl master mix to each well of the CDNA plate.
Seal and shake at 1600 rpm for 00:01:00.
Centrifuge at 1000 rpm for 00:01:00.

Set up a PCR (RABV FSM program) as follows:

Choose the preheat lid option
Set the reaction volume to 25 µL
25 °C 00:05:00

50 °C 00:10:00
80 °C 00:05:00
Hold at 4 °C

Targeted Amplification of cDNA

10m 15s

5 Preparation of RABV Primer Pools

5.1 Prepare the primer pool mix as provided in the RABV_PrimerPool sheets. Store the Rabies Primer Pools (Odd and Even Primer mix) at -20 °C

RABV_PrimerPool.xlsx 12KB

Note

1. The RABV_Primer Pools were designed using Primal Scheme (https://primalscheme.com/)

2. The RABV_PrimerPool.xlsx contains two sheets, for odd and even primal pool mix. The quantity of [M] 100 micromolar (µM) stock solution to be taken for generating a primer pool and the resulting final concentration in (uM) is provided in the the excel sheet.

5.2 Prepare two master mixes as follows.

1. Odd Rabies PrimerPool Master Mix

IPM (Illumina PCR Mix): 1260 µL
Odd PrimerPool Mix: 361.20 µL
Nuclease Free Water: 394.8 µL

2. Even Rabies PrimerPool Master Mix

IPM (Illumina PCR Mix): 1260 µL
Odd PrimerPool Mix: 361.20 µL
Nuclease Free Water: 394.8 µL

Note

The Master mix is calculated to accommodate 96 reactions. If using lesser samples calculate accordingly.

5.3 Amplification PCR

10m 15s

Label two PCR plates as
(i) RABV_Odd plate
(ii) RABV_Even Plate

Add 20 µL of Odd Rabies PrimerPool Master Mix to each well of the RABV_Odd plate
Add 5 µL of cDNA synthesised in the previous step to the corresponding well of the RABV_Odd plate.
Add 20 µL of Even Rabies PrimerPool Master Mix to each well of the RABV_Even plate
Add 5 µL of cDNA synthesised in the previous step to the corresponding well of the RABV_even plate.
Seal and shake at 1600 rpm for 00:01:00.
Centrifuge at 1000 rpm for 00:01:00.

Set up two PCR's (RABV amplification program) as follows:

Choose the preheat lid option

Set the reaction volume to 25 µL
98 °C for 00:03:00
35 cycles of:
98 °C for 00:00:15

63 °C for 00:05:00
Hold at 4 °C

Note

If you are stopping, seal the plate and store at -20 °C for up to 3 days

Tagment of PCR Amplicons

7m

6 This step uses EBLTS (Enrichment Bead-Linked Transposomes) to tagment PCR amplicons, which is a process that fragments and tags the PCR amplicons with adapter sequences.

6.1 Thaw EBLTS and TB1 Buffer at Room temperature

Note

If the RABV_Odd plate and RABV_Even Plate were stored thaw at Room temperature, shake the plates at 1600 rpm for 1 minute and centrifuge at 1000 x g for 1 minute before starting.

Label a new PCR plate as TAG.

Transfer 10 µL from each well of the RABV_Odd plate to the corresponding well of the TAG plate.
Transfer 10 µL from each well of the RABV_Even Plate to the corresponding well of the TAG plate

6.2 Prepare **Tagmentation Master Mix** in a 15 ml tube as follows. 2m

TB1 1008 µL
EBLTS 336 µL
Nuclease Free Water 1680 µL

Note

Ensure that the beads are uniformly mixed before use. Pipette mix if needed and pulse centrifuge before use.

Add 30 µl master mix to each well in the TAG plate.
Seal and shake at 1600 rpm for 00:01:00
Centrifuge at 1000 rpm for 00:01:00

6.3 Set up PCR (TAG program) as follows: 5m

Choose the preheat lid option
Set the reaction volume to 50 µl

55 °C for 00:05:00
Hold at 10 °C

Post Tagmentation Clean Up 12m

7 This step washes the adapter-tagged amplicons before PCR amplification.

7.1 Vortex ST2 (Stop Tagment Buffer 2) and TWB (Tagmentation Wash Buffer) before use. 10m

Centrifuge the TAG plate at 500 x g for 00:01:00.
Add 10 µL ST2 to each well of the TAG plate.
Seal and shake at 1600 rpm for 1 minute.
Incubate at Room temperature for 00:05:00.
Centrifuge at for 00:01:00.
Place on the magnetic stand and wait until the liquid is clear (00:03:00).

Note

If the liquid is not clear at this point continue to place it on the magnetic stand for another ~2 minutes. Inspect for bubbles on the seal. If present, centrifuge at 500 x g for 1 minute, and then place on the magnetic stand (~3 minutes).

7.2 **Wash the beads as follows:** 2m

Remove from the magnetic stand.
Add 100 µL TWB to each well.
Seal and shake at 1600 rpm for 00:01:00.
Centrifuge for 00:01:00.
Place on the magnetic stand and wait until the liquid is clear (~3 minutes).

Note

If the liquid is not clear at this point continue to place it on the magnetic stand for another ~2 minutes. Inspect for bubbles on the seal. If present, centrifuge at 500 x g for 1 minute, and then place on the magnetic stand (~3 minutes).

Remove and discard all supernatant from each well.

Wash beads a second time.
Leave supernatant in theplate for the second wash to prevent beads from overdrying.

Amplify Tagmented Amplicons and Indexing 11m 50s

8 This step amplifies the tagmented amplicons using a PCR program. The PCR step adds prepaired 10 base pair Index 1 (i7) adapters, Index 2 (i5) adapters, and sequences required for sequencing

8.1 Prepare the Master mix as follows, in a 15 ml tube.

EPM (Enhanced PCR Mix): 2016 µL
Nuclease-free water: 2016 µL

Note

Centrifuge the TAG plate, keep the on magnetic stand and remove remaining TWB. Do not leave any residual TWB in the wells.

8.2 11m 50s

Add 40 µL PCR Master Mix to each well.

Add 10 µL index adapters to each well of the PCR plate.

Seal and shake at 1600 rpm for 00:01:00

If the liquid is visible on the seal, centrifuge at 500 x g for 1 minute.

Inspect to make sure beads are resuspended.

Set up a PCR (Amplification and Indexing PCR) as follows:

Choose the preheat lid option and set to 100 °C

Set the reaction volume to 50 µL

72 °C for 00:03:00

98 °C for 00:03:00

7 cycles of:

- 98 °C for 00:00:20
- 60 °C for 00:00:30
- 72 °C for 00:01:00

72 °C for 00:03:00

Hold at 10 °C

Pool and Clean Up Libraries

8m 30s

9 This step combines libraries from each 96-well sample plate into one 1.7 ml tube. Libraries of optimal size are then bound to magnetic beads, and fragments that are too small or large are washed away.

9.1 6m

Centrifuge the TAG plate at 500 x g for 00:01:00.

Place on the magnetic stand and wait until the liquid is clear (~3 minutes).

Transfer 5 µL library from each well of the TAG plate into a 1.7 ml tube.
Vortex the tubes to mix, and then centrifuge briefly.
Add 0.9x of IPB.

Note

Assuming that 5 µL from 96 wells were pooled, give a total volume of 480 µL add 432 µL of IPB (480x 0.9).

Vortex to mix.
Incubate at Room temperature for 00:05:00.
Centrifuge briefly.

Place on the magnetic stand and wait until the liquid is clear (~5 minutes).
Remove and discard all supernatant.

9.2 Wash beads as follows. 30s

Keep on the magnetic stand and add 1000 µL fresh 80% Ethanol.
Incubate at Room temperature 00:00:30.
Remove and discard all supernatant.

Wash beads a second time.
Centrifuge briefly.
Use a 20 µl pipette to remove all residual EtOH.

Note

The final

9.3 Add 55 µL Resuspension Buffer (RSB) 2m
Vortex to mix, and then centrifuge briefly.
Incubate at room temperature for 00:02:00.
Place on the magnetic stand and wait until the liquid is clear (~2 minutes).

Transfer 50 µL supernatant to a fresh new microcentrifuge tube.

Note

The final library can be at -20 °C for up to 30 days.
We check the quality of the final library prepared using a Tapestation and the library is quantified using a Qubit Fluorometer.