

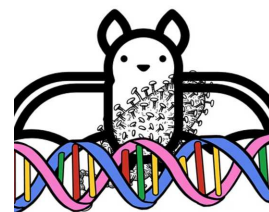
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Version 1

Rabies Virus Bat-Clade Sequencing V.1

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Abstract

Join us in advancing global genomic surveillance of the rabies virus.

We are actively engaged in pioneering a pan-clade whole genome amplicon sequencing protocol for the rabies virus. If you're interested in collaborating on this crucial endeavor and wish to obtain a primer aliquot from our established protocol or the one currently in development, please do not hesitate to contact us at richardssalvato@gmail.com.

Background

Rabies virus (RABV) causes fatal encephalitis in domestic animals and humans. This high-impact zoonotic virus is poorly studied from the genomic perspective so establishing genomic surveillance protocols for RABV is crucial to track their evolution and monitor spillover events and wildlife reservoirs.

Despite the importance of RABV zoonotic cycle, there is limited complete genome sequence data of RABVs from some hosts as bats. We developed a novel rapidly deployable, cost-effective, and flexible amplicon-based sequencing approach usable with protocols widely established during the COVID-19 pandemic and suitable to different hosts based on a one-health context. We used PrimalScheme to generate a primers panel and then aligned them with a RABV sequences dataset from different species and manually degenerated the primers to cover a wider diversity of hosts.

The set of 47 primers is compatible and ready to use with COVIDSeq sequencing protocol and Illumina DNA Prep, and allows to sequence up to 384 samples per run on the Illumina MiSeq system or to accommodate in libraries with other sample types. Additionally, we included primers for amplification of a fragment of the mitochondrial gene COI to host species identification.

COI

Initial Validation

In the initial validation, we sequenced 160 complete RABV genomes from different species from five distinct families (Bovine, Equine, Caprine, Felines, Microchiroptera) with an average coverage of 98%, most of them recovering the whole genome (88/160).

Conclusions

Here, we introduced a cost-effective and easy-to-use sequencing protocol for RABV bat-clade in order to support genomic surveillance of a re-emerging zoonotic disease allowing targeting viral control programs and adequate public health policies.

Acknowledgements

We would like to thank *Instituto de Pesquisas Veterinárias Desidério Finamor* for sample processing and initial rabies virus diagnosis.

Materials

A	B	C
primer	sequence	pool
raiva__1_LEFT	ATGTGGAAGGRARTTGGGCTCT	1
raiva__1_RIGHT	TTGACKGTTCCGTCATCTGCC	1
raiva__2_LEFT	CATGAGATGTCWGTCTTGGRGG	2
raiva__2_RIGHT	GTGACATAGGATATGATCTCCTCRAC	2
raiva__3_LEFT	AGATTTTTGTCARYCCAAGTGCG	1
raiva__3_RIGHT	CATCTCAGAGGGAGYTTGGATCC	1
raiva__4_LEFT	TGAARATGAACCTTGAYGACATAGTC	2
raiva__4_RIGHT	CATGTTTRATACACCAAATYCTKCC	2
raiva__5_LEFT	GAACTGGGTATAYAARTTGAGGAGAAC	1
raiva__5_RIGHT	CTTGAATGTGGTRGTGACATAACC	1
raiva__6_LEFT	AAGGTGGGRTACATMTCYGCCA	2
raiva__6_RIGHT	TATGCCTTYCCAAAYCCMGG	2
raiva__7_LEFT	ACYGTAAARACCACYAARGAGT	1
raiva__7_RIGHT	ACAGARGACTCYARCAGCTCCA	1
raiva__8_LEFT	TRATGAKTGCAGGTGSTCTGG	2
raiva__8_RIGHT	CTTAAGATRTTGGGRAYGGYGGG	2
raiva__9_LEFT	RACAGACAAYTGYTCKAGGTCWTACA	1
raiva__9_RIGHT	CCTCMAGYTGACYCACCTTRTCYC	1
raiva__10_LEFT	CCTTGAYTGGGATGARGAGAA	2
raiva__10_RIGHT	TGTTTGGGAGGCCAYGTYTG	2
raiva__11_LEFT	CYAAATGGTATCTTGATYCGCGAC	1
raiva__11_RIGHT	YTGACATGTCCAGACARTATATTTGATC	1
raiva__12_LEFT	TCTGTACTYGATCAAGTGTTYGGA	2



A	B	C
raiva_12_RIGHT	GARAACTGACGTATRTGGAACCTT	2
raiva_13_LEFT	GCTGTMTTCCATTACYTGCTST	1
raiva_13_RIGHT	CCCAACATYTCAGAGGGRTGRG	1
raiva_14_LEFT	SATGACMCAGACTCCCCAAAGG	2
raiva_14_RIGHT	GCACDGCTCCMGAACCATTTCTA	2
raiva_15_LEFT	TTGGCATCTTMGATGTAACAAGTG	1
raiva_15_RIGHT	GARCTCATTTGTCKYAAGTTGG	1
raiva_16_LEFT	TCWGACTTTAGAAGYTCYAAGATGAC	2
raiva_16_RIGHT	GTGACCTCHGCATCACAAATGA	2
raiva_17_LEFT	TGATGGCRTCAGGRACACAYC	1
raiva_17_RIGHT	CTGCAGCATATGTTGAAGTGTCTC	1
raiva_18_LEFT	GYTDATGTCTGATTTTGCAYTRTC	2
raiva_18_RIGHT	TCARCCTGATCCAGTGAGAWGA	2
raiva_extra_1_LEFT	ACGCTTAACRACAAAATCAG	2
raiva_extra_1_RIGHT	ATGTTTGTCTTGTAATTGCC	2
raiva_extra_2_LEFT	ATATTCAACAAGACYTTRAT	2
raiva_extra_2_RIGHT	GTACAACTCCCATGARGATA	2
raiva_extra_3_LEFT	CGAYTTGCCTCCTATGAAGG	1
raiva_extra_3_RIGHT	AGCCAAAGGGAGATCATMGA	1
raiva_extra_4_LEFT	TACAACAGACCCATAACYTA	1
raiva_extra_4_RIGHT	ACGCTTAACAAAAAACAATAAAGAT	1
raiva_extra_5_RIGHT	ACTTGGAACGAGATCATCCC	2
raiva_extra_5_LEFTA	CCTATGAAGGACACAAGCAA	2
raiva_extra_5_LEFTB	CCTATGAAGGACCCTAGCAA	2
Mod_RepCOI_F	TNTTYTCMACYAACCACAAAGA	1
Mod_RepCOI_R	TTCDGGRTGNCCRAARAATCA	2
VertCOI_7194_F	CGMATRAAYAAYATRAGCTTCTGAY	2

	A	B	C
	VertCOI_7216_R	CARAAGCTYATGTTRTTYATDCG	1

Primers sequences and respective pools



Primer Preparation

- 1 Reconstitute each primer shown in Table 1 (See Materials section), using nuclease-free water to get a 100 μ M stock solution.
- 2 Prepare RABV-BAT primer pools A and B as described here.
 - 2.1 Separate all primers at 100 μ M into two separate boxes labeled as Pool A and Pool B, according to Table 1.
 - 2.2 Label a 2.0 ml microtube as Pool A and another as Pool B.
 - 2.3 Vortex and spin down all the primers tubes.

Add  5 μ L of each 100 μ M primer from Pool A into the tube labeled as Pool A.

Add  5 μ L of each 100 μ M primer from Pool B into the tube labeled as Pool B.
 - 2.4 Add  1080 μ L of nuclease-free water into the tube with Pool A.

Add  1215 μ L of nuclease-free water into the tube with Pool B.

Now you have Pool A and B at a concentration of 10 μ M and ready to use.
 - 2.5 Pooled Primers Can Be Stored at  -20 $^{\circ}$ C

Sample Extraction and Cycle threshold (Ct) determination

28m 45s

- 3 Samples were extracted as described below and we used a previously published Real-time RT-PCR on all samples to determine viral load with Cycle threshold (Ct) value. Samples with a Ct value <28 are recommended for optimal results.
 - 3.1 A total of  200 μ L of homogenate samples, containing  50 mg of brain tissue fragments macerated in Basal Medium Eagle (BME), were added to  800 μ L of



TRIzolTM Reagent (Invitrogen, Grand Island, NY, USA) in 2.0 ml microtubes.

3.2 The tissue was disrupted using a vortex at maximum speed for 00:00:15 , followed by Room temperature incubation for 00:05:00 and then 180 μ L of chloroform was added. 5m 15s

3.3 The tissue was disrupted using a vortex at maximum speed for 00:00:15 , followed by Room temperature incubation for 00:05:00 and then 180 μ L of chloroform was added. 5m 15s

3.4 The mixture was mixed for 00:00:15 , incubated at Room temperature for 00:03:00 , and centrifuged at 12000 rcf at 4 $^{\circ}$ C for 00:15:00 . 18m 15s

3.5 Viral RNA extraction was carried out using 200 μ L of supernatant using a commercially available Extracta Kit Fast – DNA e RNA Viral (MVXA-PV96-B FAST) and the Loccus Extracta® 96 equipment (Loccus, Sao Paulo, Brazil) following the manufacturer's instructions. Extracted RNA samples were stored at -80° C until tested by RT-qPCR.

3.6 For Cycle threshold (Ct) determination TaqMan single-step RT-PCR assay was conducted according previously described by Crystal Gigante (See protocol below).

Protocol

NAME

LN34 pan-lyssavirus real-time RT-PCR for post-mortem diagnosis of rabies in animals

CREATED BY

Crystal Gigante

Preview

4 cDNA synthesis and amplification of amplicons can be performed using the Illumina COVIDSeq Test (RUO version) or Illumina DNA Prep. Choose one of these approaches below.

STEP CASE

Illumina COVIDSeq Test (RUO version)

27 steps



5 This step reverse transcribes the RNA fragments primed with random hexamers into first-strand cDNA using reverse transcriptase.

* Include a negative PCR control (NTC; nuclease-free water) for each pool.

5.1 Label new PCR plate CDNA1.

5.2 Add  8.5 µL EPH3 to each well.

5.3 Add  8.5 µL eluted sample to each well.

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

1m

5.5 Centrifuge at 1000 × g for 1 minute.

5.6 Place on the preprogrammed thermal cycler and run the COVIDSeq Anneal program.

COVIDSeq Anneal program:

- Choose the preheat lid option
- Set the reaction volume to 17 µl
- 65°C for 3 minutes
- Hold at 4°C

5.7 In a 1.7 ml tube, combine the following volumes to prepare First Strand cDNA Master Mix.

*Multiply each volume by the number of samples.

FSM  9 µL

RVT  1 µL

Reagent overage is included to account for small pipetting errors.

5.8 Add  8 µL master mix to each well of the CDNA1 plate.



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

1m

5.10 Centrifuge at  1000 x g for  00:01:00 .

1m

5.11 Place on the preprogrammed thermal cycler and run the COVIDSeq FSS program.

COVIDSeq FSS program:

- Choose the preheat lid option
- Set the reaction volume to 25 µl
- 25°C for 5 minutes
- 50°C for 10 minutes
- 80°C for 5 minutes
- Hold at 4°C

Amplicon Generation

6 This step uses two separate PCR reactions to amplify cDNA using the previously prepared primers pool A and B.

* Include a negative PCR control (NTC; nuclease-free water) for each pool.

7 Label two new PCR plates POOL A and POOL B. The plates represent two separate PCR reactions.

8 In a 15 ml tube, combine the following volumes to prepare PCR 1 Master Mix and PCR 2 Master Mix. Multiply each volume by the number of samples.

*Reagent overage is included to account for small pipetting errors.

	A	B	C
	Reagent	PCR 1 Master Mix (µl)	PCR 2 Master Mix (µl)
	IPM	15	15
	RABV-BAT Pool A	4.3	N/A
	RABV-BAT Pool B	N/A	4.3



	A	B	C
	Nuclease-free water	4.7	4.7

9 Add  20 μL PCR 1 Master Mix to each well of the POOL A plate corresponding to each well of the CDNA1 plate.

9.1 Add  5 μL first strand cDNA synthesis from each well of the CDNA1 plate to the corresponding well of the POOL A plate.

9.2 Add  20 μL COVIDSeq PCR 2 Master Mix to each well of the POOL B plate corresponding to each well of the CDNA1 plate.

9.3 Add  5 μL first strand cDNA synthesis from each well of the CDNA1 plate to the corresponding well of the POOL B plate.

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

1m

9.5 Centrifuge at  1000 x g for  00:01:00 .

1m

9.6 Place in the preprogrammed thermal cycler and run the COVIDSeq PCR program.

COVIDSeq PCR program:

- Choose the preheat lid option
- Set the reaction volume to 25 μL
- 98°C for 3 minutes
- 35 cycles of:
 - 98°C for 15 seconds
 - 63°C for 5 minutes
- Hold at 4°C

10 *Safe Stopping Point:



Amplicons can be stored at  -20 °C until ready to use

Amplicon checking

1m

- 11 We recommend using agarose gel electrophoresis or automated electrophoresis to check the amplicons before proceeding to library construction.

Library preparation

- 12 **The following steps were conducted according to the standard protocol, starting from "Tagment PCR Amplicons" step on page 9.**

 illumina-covidseq-ruo-kits-referen... 785.7KB

Tagment PCR Amplicons
Post Tagmentation Clean Up
Amplify Tagmented Amplicons
Pool and Clean Up Libraries
Quantify and Normalize Libraries
Pool and Dilute Libraries
Prepare for Sequencing

Sequencing

- 13 The sequencing can be performed in any Illumina platform or flowcell using 2×150 nt reads.

Note

For sequencing, we recommend generating at least 50,000 reads per sample or 100,000 reads for optimal sequencing coverage.

Bioinformatics Analysis

- 14 Sequencing reads should be analyzed by an amplicon-based sequencing pipeline. We recommend using ViralFlow Workflow which performs several genomic analyses based



on reference genome assembly. See details at <https://viralflow.github.io/>

From our experience, better coverage and depth are obtained when using subclade-specific references. So, we usually assembly the sequences using the bat clade reference (JQ685956), then we run the consensus resulting sequence at <http://rabv-glue.cvr.gla.ac.uk/> typing tool, and getting the nearest reference we re-assembly using this reference genome.

Reference Sequence

 JQ685956.fasta 11.9KB

BED file

 JQ685956.bed 1.3KB

COI Analysis

COI amplicon analysis can be performed using [ampliseq](#) pipeline, an amplicon sequencing analysis workflow using DADA2 and QIIME2.