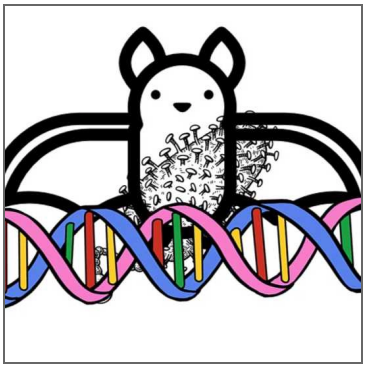


VERSION 2

MAR 09, 2024

OPEN ACCESS

DOI:
dx.doi.org/10.17504/protocols.io.8epv5x3bng1b/v2

Protocol Citation: Fernanda Godinho, Aline Campos, rosana-huff, Amanda Ruivo, Milena Bauermann, Thales Bermann, Gabriel Luz Wallau, Paulo Michel Roehe, Richard Salvato 2024. Rabies Virus Bat-Clade Sequencing. **protocols.io** https://dx.doi.org/10.17504/protocols.io.8epv5x3bng1b/v2Version created by Richard Salvato

Rabies Virus Bat-Clade Sequencing V.2

Fernanda Godinho[1], Aline Campos[1], rosana-huff[1], Amanda Ruivo[1], Milena Bauermann[1], Thales Bermann[1,2], Gabriel Luz Wallau[3], Paulo Michel Roehe[1,2], Richard Salvato[1,2]

[1]Secretaria Estadual da Saúde do Rio Grande do Sul; [2]UFRGS; [3]FIOCRUZ

Richard Salvato
UFRGS

DISCLAIMER

This sequencing protocol is shared informally, and its use is at the discretion of the recipient. We make no guarantees or warranties, express or implied, regarding the accuracy, completeness, or fitness for any particular purpose of this protocol.

The information provided is intended solely for informational and collaborative purposes. Users are solely responsible for conducting their own due diligence, quality control, and validation to ensure that the protocol aligns with their specific research objectives and adheres to all applicable legal and ethical guidelines. Any reliance on the protocol is at the user's own risk. This protocol does not constitute professional advice, and any application or implementation should be carried out by appropriately trained and qualified personnel.

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

Created: Mar 07, 2024

Last Modified: Mar 09, 2024

PROTOCOL integer ID: 96309

Keywords: rabies, viruses, genomics, sequencing

Funders Acknowledgement:
FAPERGS
Grant ID: 23/2551-0000510-7
FAPERGS
Grant ID: 23/2551-0000852-1
FAPERGS/CNPQ
Grant ID: Programa de Apoio à Fixação de Jovens Doutores no Brasil

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	D
	primer	sequence	pool	
	raiva__1_LEFT	ATGTGGAAGGRARTTGGGCTCT	1	
	raiva__1_RIGHT	TTGACKGTTCCGTCATCTGCC	1	
	raiva__2_LEFT	CATGAGATGTCWGTTCTTGGRGG	2	
	raiva__2_RIGHT	GTGACATAGGATATGATCTCCTCRAC	2	
	raiva__3_LEFT	AGATTTTTGTCARYCCAAGTGCG	1	
	raiva__3_RIGHT	CATCTCAGAGGGAGYTTGGATCC	1	
	raiva__4_LEFT	TGAARATGAACCTTGAYGACATAGTC	2	
	raiva__4_RIGHT	CATGTTRATACACCAAATYCTKCC	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	CCTTGGAYTGGGATGARGAGAA	2	
	raiva__10_RIGHT	TGTTTGGGAGGCCAYGTYTG	2	
	raiva__11_LEFT	CYAAATGGTATCTTGATYCGCGAC	1	
	raiva__11_RIGHT	YTGACATGTCCAGACARTATATTTGATC	1	
	raiva__12_LEFT	TCTGTACTYGATCAAGTGTTYGGA	2	
	raiva__12_RIGHT	GARAACTGACGTATRTGGAACCTT	2	
	raiva__13_LEFT	GCTGTMTTCCATTACYTGCTST	1	
	raiva__13_RIGHT	CCCAACATYTCAGAGGGRTGRG	1	
	raiva__14_LEFT	SATGACMCAGACTCCCCAAAGG	2	
	raiva__14_RIGHT	GCACDGCTCCMGAAACCATTCTA	2	

	A	B	C	D
	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	ACGCTTAACAAAAAAACAATAAAGAT	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	
	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 µM stock solution.

2 Prepare RABV-BAT primer pools A and B as described here.

2.1 Separate all primers at 100 µM 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 µL of each 100 µM primer from Pool A into the tube labeled as Pool A.
Add 5 µL of each 100 µM primer from Pool B into the tube labeled as Pool B.

2.4 Add 1080 µL of nuclease-free water into the tube with Pool A.
Add 1215 µL of nuclease-free water into the tube with Pool B.

Now you have Pool A and B at a concentration of 10 µM and ready to use.

2.5 Pooled Primers Can Be Stored at -20 °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 µL of homogenate samples, containing 50 mg of brain tissue fragments macerated in Basal Medium Eagle (BME), were added to 800 µL 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 5m 15s Room temperature incubation for 00:05:00 and then 180 µL of chloroform was added.

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

3.4 The mixture was mixed for 00:00:15, incubated at Room temperature for 18m 15s 00:03:00, and centrifuged at 12000 rcf at 4 °C for 00:15:00.

3.5 Viral RNA extraction was carried out using 200 µL 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

cDNA Synthesis

1m

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
	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-reference-gui… 785KB

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 2x150 nt reads.

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

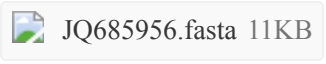
Bioinformatics Analysis

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/

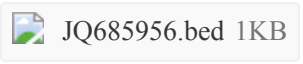
From our experience, better coverage and depth are obtained when using subclade-specific references. So, we usually assembly the sequences using the bat clade reference (JQ685956), then we run the consensus

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



BED file



COI Analysis

COI amplicon analysis can be performed using ampliseq pipeline, an amplicon sequencing analysis workflow using DADA2 and QIIME2.