Sanger sequencing of lyssaviruses

Version 01

April 2021

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FOREWORD

This protocol has been adapted by:

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INTRODUCTION

This Standard Operating Procedure (SOP) describes the Sanger sequencing of lyssaviruses. Sanger sequencing also known as the "chain termination method" is based on the detection of labelled chain-terminating nucleotides that are incorporated by a DNA polymerase during the replication of the template submitted to the analysis. This method developed in 1977 by Frederick Sanger and his colleagues is the "first-generation" DNA sequencing method and has been extensively used for more than 40 years across the world. Despite the advantages of the next-generation sequencing (NGS), Sanger sequencing is still widely used, particularly when partial genome characterization is sufficient for virus typing or for sequencing of single genes and in some cases for the analysis of longer fragments (~1000 bp in length).

This protocol should not be considered transferable as it stands. It shall be adapted by each user for its working conditions and shall be validated with reference materials or samples of known status (as performed in the frame of rabies diagnosis proficiency testing by example).

All commercial references described in this SOP are given for information. Other equivalent reagents, or equipment could be used as far as it does not affect the results.

1. PURPOSE AND SCOPE

This SOP describes the preparation of PCR products from lyssavirus-positive samples for the characterization of the lyssavirus species. Many national and international service providers with competitive costs carry out routinely Sanger sequencing and usually achieved the DNA sequencing results at D+1. This SOP will therefore be limited to the preparation of PCR products for sending and sequencing by a service provider for the characterisation of lyssavirus species.

The SOP is based on a conventional (gel-based) PCR assay and the use of modified sequencing primers. The primers are standard primers classically used for Sanger sequencing (M13uni-29; M13rev-49) combined with pan-Lyssavirus primers previously described by Heaton et al. [2] for the detection of all ICTV recognized lyssavirus species.

2. REFERENCE DOCUMENTS

1. OIE. Chapter 3.1.17. Rabies (Infection with rabies virus and other Lyssaviruses). Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2019 Paris, France;World Organisation for Animal Health.
2. Heaton, P.R., Johnstone, P., McElhinney, L.M., Cowley, R., O'Sullivan, E. and Whitby, J.E., 1997. Heminested PCR assay for detection of six genotypes of rabies and rabies-related viruses. J Clin Microbiol 35, 2762-6.
3. Darryl L. Davis, Edward P. O'Brie, and Catherine M. Bentzley. Analysis of the degradation of oligonucleotide strands during the freezing/thawing processes using MALDI-MS. Anal Chem 2000 Oct 15;72(20):5092-6.

3. TERMS, ABBREVIATIONS AND DEFINITIONS

bp	base pair
RT-PCR	reverse transcriptase polymerase chain reaction
ICTV	International Committee on Taxonomy of Viruses
N	Nucleoprotein gene
PCR	PCR
RABV	Classical rabies virus
RNA	Ribonucleic Acid

4. REAGENTS[1]

- RT-PCR kit: Qiagen One step RT-PCR kit (Qiagen, France)
- Nested PCR: Platinium Taq polymerase and its associated buffer (Invitrogen, France)
- RNAse Out 40 U/µl
- dNTP 100mM
- Molecular grade water DNAse-RNase free
- Electrophoresis: Loading dye buffer, agarose, nucleic acid gel staining

5. EQUIPMENT AND MATERIALS

5.1. EQUIPMENT

- Dedicated and separated laboratory rooms or workstations for the preparation of primers/master mix, extraction of RNA, PCR amplification and post PCR
- PCR workstations with specific dedicated micropipettes
- Spin micro-centrifuges, refrigerated centrifuges
- Vortex
- Electrophoresis apparatus
- Conventional thermocycler

5.2. CONSUMABLES

- DNAse-RNase free, sterile and single use plastic consumables: microtubes, filter tips, …
- Single use gloves

[1] All commercial references described in this document are given for information. Other equivalent reagents or equipment can be used. Each user must validate its protocol in its working conditions (reagents, equipment).

5.3. PRIMERS

The molecular models used for the characterization of the Lyssavirus species are described in the Table below:

Molecular models used:

Primers	Function	Sequences (5'-3')	Localisation	Usage	Target
JW12	F	ATGTAACACCYCTACAATG	55-73	RT-PCR	Lyssavirus
JW6.1	R	CAATTCGCACACATTTTGTG	660-641	RT-PCR	Lyssavirus
JW6.2		CAGTTGGCACACATCTTGTG			
JW6.3		CAGTTAGCGCACATCTTATG			
$M13^{(1)}$-JW12	F	TGTAAAACGACGGCCAGTATGTAACACCYCTACAATG	/	PCR sequencing	Lyssavirus
$M13^{(2)}$-JW6	R	GGATAACAATTTCACACAGGCARTTCGCACACATTTTRTG			

F: forward; R: reverse; $M13^{(1)}$: M13uni-21; $M13^{(2)}$: M13rev-49

All the masters stocks are at 100µM, aliquoted and stored at < -16°C. Working primers are at 20µM, aliquoted and stored at < -16°C.

Repeated freeze/thaw cycles should be avoided [7]. In the EURL, the number of freezing / thawing is limited to 5 times in order to preserve the characteristics of primers.

6. PROCEDURE

Follow the usual recommendations to avoid any contamination.

6.1. RT-PCR

6.1.1. PREPARATION OF THE MASTER MIX[2]

1. Thaw all frozen reagents (i.e. Qiagen One step RT-PCR, primers, molecular grade water, dNTPs). Mix the individual solutions and place them on ice until the addition of the enzyme mix.
2. Prepare the master mix as follows:

Master mix	Final concentration	Volume (in µL) for 1 tube
Molecular grade water		4.25
Qiagen One step RT-PCR (5X)	1X	3
dNTPs (10mM)	0.4mM	0.6
JW12 (20µM)	0.7	0.525
JW6 (20µM of each primer)	2	0.525
Qiagen RT-PCR Ez mix		0.6
RNAse Inibitor (40 U/µl)	20 U	0.5

3. Mix the reaction thoroughly.
4. Dispense a volume of 10µL of master mix into PCR tubes.

6.1.2. ADDITION OF TEMPLATE

5. Add 5µL of RNA samples/control RNA(s) to the individual PCR tubes containing the master mix and close the tubes.
6. Transfer the PCR tubes to the thermocycler for thermal cycling.

For each run, include positive and negative controls as follows:

- A **PCR negative control** = ultrapure water instead of sample to validate the no-contamination of the PCR.
- A **PCR positive control** = known target RNA previously extracted and included to ensure the good running of PCR.

[2] There are numerous available commercially reagents dedicated for RT-PCR and PCR. Optimisation of PCR (master mix formulation and cycling parameters) should be carried out by each user according to the enzyme mix used and the thermocycler used under his own working conditions.

6.1.3. SETTING UP OF THERMOCYCLER[3]

7. Load the samples into the machine.
8. Program the thermocycler as follows:

STEP	Temperature	Time	Number of cycles
Reverse transcription	50°C	30 min	1
Polymerase activation	95°C	15 min	1
Denaturation	94°C	30 sec	
Annealing	55°C	30 sec	45x
Extension	72°C	1 min	
Final extension	72°C	10 min	1

Storage: Lyssavirus RT-PCR products are used to implement the sequencing PCR. They are stored at 5±3°C if used during the day or at <-16±3°C for longer storage.

6.2. SEQUENCING PCR

6.2.1. PREPARATION OF THE MASTER MIX

9. Mix the individual solutions and place them on ice.
10. Prepare the master mix as follows:

Master mix	Final concentration	Volume (in µL) for 1 tube
Molecular grade water		14.45
PCR Buffer without $MgCl_2$ (10X)	1X	2
$MgCl_2$ (50mM)	1.5mM	0.6
dNTPs (10mM)	0.1mM	0.2
JM13-JW12 (20µM)	0.25	0.25
M13-JW6 (20µM of each primer)	0.25	0.25
Taq Platinium (5U/µl)	1.25U	0.25

11. Mix the reaction thoroughly.
12. Dispense a volume of 18µL of master mix into PCR tubes.

[3] The cycling parameters have been validated on the conventional thermocycler Eppendorf Mastercycler Gradient. Thermal profiles may be subject to optimization depending on used PCR equipment and kits.

6.2.2. ADDITION OF TEMPLATE

13. Add 2µL of the RT-PCR Lyssavirus products to the individual PCR tubes containing the master mix and close the tubes.
14. Transfer the PCR tubes to the thermocycler for thermal cycling.

6.2.3. SETTING UP OF CONVENTIONAL THERMOCYCLER

15. Load the samples into the machine.
16. Program the thermocycler as follows:

STEP	Temperature	Time	Number of cycles
Polymerase activation	95°C	2 min	1
Denaturation	94°C	30 sec	30x
Annealing	61°C	30 sec	
Extension	72°C	30 sec	
Final extension	72°C	10 min	1

17. Transfer the PCR tubes to a room dedicated to electrophoresis.

Storage: PCR products are stored at a temperature of 5±3°C until the realization of the electrophoresis then at <-16°C ±3°C before sending to the sequencing provider.

6.3. ELECTROPHORESIS

5 µL of PCR products are added to 1 µL of loading dye buffer (loading buffer is 6x concentrated). The mixture is then applied to a 1.5% agarose gel, to which an intercalant[4] has been added.

The nucleic acid gel stain used in the EURL is the SYBR safe at a final concentration of 1:10.000 (i.e. 1µl SYBR Safe per 10ml of agarose gel).

A well is reserved for the deposition of a DNA size marker in order to estimate the size of amplicons.

6.4. ANALYSIS OF PCR PRODUCTS

The result is positive when a DNA fragment is detected at the expected size of 644 bp (M13-JW12/M13-JW6).

[4] Ethidium bromide has been used for decades to stain nucleic acids in agarose gels under UV light. However, because ethidium bromide has been shown to be mutagenic and potentially carcinogen, the safer SYBR-based options have been preferred by many users over the use of ethidium bromide.

The result is negative when no DNA fragments are visible for the nested PCR Lyssavirus.

7. RESULTS

7.1. TEST VALIDATION

The tests are validated as follows:

- if the positive control = positive.
- if the negative control = negative.

7.2. SEQUENCING OF PCR PRODUCTS

Once the test is validated, the PCR products are sent to a service provider for SANGER sequencing by following the recommendations of the service provider.

Sequencing must be performed in both the reverse and forward direction (i.e. using forward M13-JW12 and reverse M13-JW6 primers for this SOP) to obtain a consensus sequence. Sequencing in each direction should be performed to reduce the likelihood of errors in sequences.

The PCR products should be purified prior to sequencing. Usually, the service provider carry out purification of PCR products (usually, free of charge).

DNA concentration and volume should respect the service provider's recommendations. DNA quantification can be estimated via agarose gel or a photometer to ensure accurate results.

An example of volume and sample concentration recommended by a service provider is shown for information below:

- Plasmid DNA with a product length<30kbp:
    - Recommended sample concentration : 50-100 ng/µL,
    - Volume of 15µL for 1-4 reactions
- Purified PCR products:
    - 150-300bp: 1 ng/µL
    - 300-1000bp: 5 ng/µL
    - 1000-3000bp: 10 ng/µL
- Unpurified products:
    - 150-300bp: 4 ng/µL
    - 300-1000bp: 10 ng/µL

- 1000-3000bp: 20 ng/µL

7.3. ANALYSIS OF DNA SEQUENCING RESULTS

The raw data can be provided in different formats depending on the model of sequencer used. Usually, the service provider provides a chromatogram, a sequence in the format ".fasta" and a sequence in the format ".ab1" or ".seq" from which we can analyse and derive the sequence.

In the EURL, the analysis of data and the generation of consensus sequence is performed using the Geneious Prime software.

Numerous software packages exist for the analysis of sequences as well as the generation of consensus sequences (e.g. Vector NTI from Invitrogen, Seqman in DNASTAR Lasergene).

The consensus sequence from the forward sequence and the reverse sequence should be performed only if the chromatogram of both forward and reverse sequences are of good quality.