Rabies virus (RABV) detection & quantitation by qRT-PCR (SYBR green).

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This protocol describes the real-time polymerase chain reaction with reverse transcription (qRT-PCR) approach developed for the detection and quantitation of viral titres in biological specimens using a either a two step (separate RT and PCR reactions) or one-step SYBR-Green modality (but easily TaqMan adaptable). RABV is a group V ((-)ssRNA) and belong to the Mononegavirales order, Rhabdoviridae family, Lyssavirus genus. These viruses are enveloped and have a single stranded negative-sense RNA genome bound into a helicoidal ribonucleoprotein complex. The genome encodes five genes of conserved order for nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and the viral RNA polymerase (L). The complete genome sequences range from 11,615 to 11,966 nt in length. Upon infection, all transcription and replication events take place in a 2–10 µm cytoplasmic vacuole called the Negri body. Lyssaviruses are characterized by an extremely broad host spectrum ranging from plants to insects and mammals; human-infecting viruses more commonly icosahedral in symmetry and take shapes approximating regular polyhedra.

Oligonucleotide primers

Name	Sequence[1]	bp	%GC	Tm	Hair	HomD	HetD	Amplicon
Lyssa-F	5´-ATg-TAA-CAC-CTC-TAC-AAT-g-3´	19	36.8	46.5	0.23	-4.26	5.77	111
Lyssa-R	5´-gCM-ggR-TAY-TTR-TAY-TCA-TA-3´	20	37.5	47.7	2	-8.19		

Note 1: Reverse oligonucleotide primer sequences given in this table are the reverse-complement of sequence present in alignments and as they should be ordered for synthesis; Hairpin (Hair), homodimer (homD) and heterodimer (HetD) DQ's given in kcal/mol.

Oligonucleotide primer map in artificial gene[2] (underlined primer sites)

AAGCTCCGCGTCCTTTACCAAGGAAATAACATCACTGTAACTGCCTATGCAAACGGCGACCATGCCGTCACAGTTAAGGACGCCA
AATTCATTGTGGGGCCAATGTCTTCAGCCTGGACACCTTTCGACAACAAAATTGTGGTGTACAAAGGTGACGTCTATAATATGGA
CTACCCGCCCTTTGGCGCAGGAAGACCAGGACAATTTGG|CAGACCACGCTACGGCGTGCTACTCTGCGGAGAGTGCAGTCTGCG
ATAGTGCCCCAGGAGGACTGGGTTAACAAAGGCAAACCAACGCCCCACGCGGCCCTAG|AAGGACTAGAGGTTAGAGGAGACCAT
GTCCATGTCACCCACGGTCATCATCCTGGCATGTCTTGGGTTCTATCCTGCTGTCTCTACAGCATCATTCCAGGCACAGAACGCC
|AAGGACTAGAGGTTAGAGGAGACCCCCCCAACACAAAAACAGCATATTGACGCTGGGAAAGACCAGAGATCCTGCTGTCTCTGC
AACATCAATCCAGGCACAGAGCGCC|AAATACACATACCAAAACAAAGTGGTGAAGGTTCTCAGACCAGCTGAAGGAGGGAAAAC
AGTCATGGACATCATCTCAAGACAAGACCAGAGAGGGAGTGGA|ATGTATGTGAGTGCTGATGCCACGAAATGGTCACCAGGAGA
TAATTCGGCAAAGTTTAAGAGATTCACACAGGCATTATATGATGGCTTGTCAGATGAGAAGTTAAAATGTTGCGTTGTTGATGC|
ATGTAACACCTCTACAATGGATGCCGACAAGATTGTATTCAAAGTCAATAATCAGGTGGTCTCTTTGAAGCCTGAGATTATCGTG
GATCAATATGAGTACAAGTACCCTGC|GCATAGCTGTAGGAAGGACTCTCAAGGCATTTTTCTCCTGGTCGCTTACTGACCCTTT
AGGGAACGAAGCCCCTGGGGGATACTGTCTTGAAAAGTGGATGCTT|GCAACGCGCGATTCAGTTCCTCTTCACATAATCGCCCC
GAGCTCGCTTATCGTTTAAGCAGCTCTGCGCTACTATGGGTCCCGTGTAGAGGC|TGATGATGCCGTCGTGTGCTACAACAGTAA
CTATGCGGCTCAAGGTTTAGTAGCTAGCATTAAGAACTTTAAGGCAGTTCTGTATTATCAAGATAATGTGTTCATGTCTGAGGCA
AAATGTTGGACTGAGACTGACCTTACTAAAGGACCTCACGAATTTTGCTCACA

Note 2: Primer sites for different pathogen sequences shown in red, TaqMan probe sites shown in blue, RABV specific primer binding sites shown in grey highlight. RABV artificial gene sequence derived from Pasteur PV strain nucleoprotein gene sequence as available from GenBank through accession number X03673.

Two-step (separate RT and PCR) components and conditions

Dual (separate RT and PCR) uses M-MULV RT Enzyme (Vivantis Cat. No.: ME2305) and iTaq qPCR Master Mix (Biorad Cat. No.: 1725150).

First strand synthesis (RT)

	cf	1x
dH_2O	---	2.5 µL
10 µM Forward oligo	1.125 µM	2.3 µL
10 µM Reverse oligo	1.125 µM	2.3 µL
10 mM dNTPs 10 mM	250 µM	0.5 µL
RNA	---	10 µL
		vf: 17.6 µl

↓

Run RT-1 program in Axygen TC-1 →

Total time: 6 min		
95 ºC	2 min	1 cycle
4 ºC	2 min	

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RT Buffer	1x cf	2 µL
RT Enzyme 250 IU/µl	5 IU/µL	0.4 µL
		vf: 20 µl

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Run RT-2 program in Axygen TC-1 →

Total time: 1:12 hrs		
38 ºC	60 min	1 cycle
95 ºC	5 min	
4 ºC	5 min	

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Polymerase Chain Reaction (PCR)

	cf	1x
dH_2O	---	3.4 µL
2x iTaq SYBR Green	1 x	5 µL
10 µM Forward oligo	300 nM	0.3 µL
10 µM Reverse oligo	300 nM	0.3 µL
Template	10 ng	1 µL
		vf: 10 µl

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Run generic program in Applied Biosystems 7500 →

Total time: 2:10 hrs		
95 ºC	3 min	
94 ºC	15 sec	40 cycles
50 ºC[3]	60 sec	
95 ºC	15 sec	
60 ºC	20 sec	
Ramp[3]	60 min	
95 ºC	15 sec	

Note 3: Data acquisition.

One-step qRT-PCR components and conditions

The One-Step qRT-PCR procedure uses VeriQuest SYBR Green One-Step qRT-PCR Mix (USB Affymetrix Cat. No.: 75700).

qRT-PCR		1x
dH_2O	cf	3.3 µL
qRT-PCR Master mix	1 x	5 µL
RT Enzyme mix	1 x	0.1 µL
10 µM Forward oligo	300 nM	0.3 µL
10 µM Reverse oligo	300 nM	0.3 µL
Template	< 1 µg	1 µL
		vf: 10 µl

↓

Run Affymetrix program in Applied 7500 ➔

Total time: 2:00 hrs		
50 ºC	10 min	
95 ºC	10 min	
95 ºC	15 sec	40 cycles
50 ºC[3]	30 sec	
95 ºC	15 sec	
60 ºC	20 sec	
Ramp[3]	60 min	
95 ºC	15 sec	

Note 3: Data acquisition.

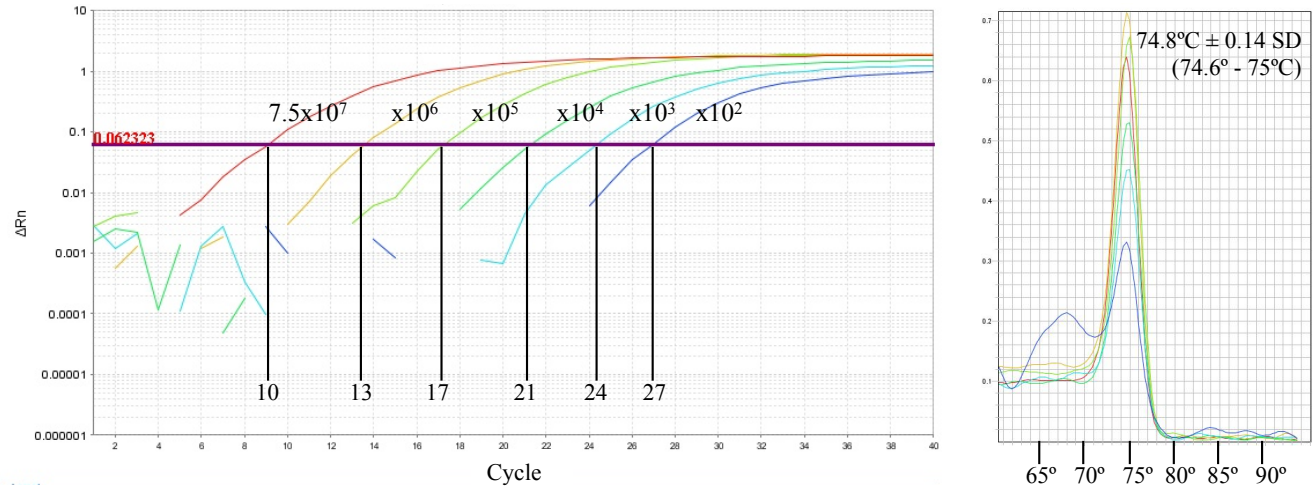
Titration curve preparation for quantitative analysis of viral titres

Using Artificial Gene (AG) sequence stock having all viral references (CHIKV, WNV, DENV1-3, DENV2-4, ZIKV, SNV, RABV, TCRV, MERS and SARS) prepare the following log titres (stock currently at $7.51x10^7$ cp/µL, working dilution of AG prepared at $7.51x10^6$ cp/µL).

		AG stock	dH_20 vol	Ct mean
1st	$7.5x10^7$ cp/µL	10 µL	90 µL	9.68 (±0.75)
2nd	$7.5x10^6$ cp/µL	10 µL	90 µL	13.46 (±0.4)
3rd	$7.5x10^5$ cp/µL	10 µL	90 µL	17.16 (±0.41)
4th	$7.5x10^4$ cp/µL	10 µL	90 µL	21.13 (0.21)
5th	$7.5x10^3$ cp/µL	10 µL	90 µL	24.43 (±0.28)
6th	$7.5x10^2$ cp/µL	10 µL	90 µL	26.70 (±0.26)

Add 90 µL to each of the 6 PCR 0.2 mL tubes. Take 10 µL of initial working stock (at $7.51x10^7$ cp/µL) and dispense into 1st PCR tube, wash tip 30 times, cap, vortex for 10 seconds and spin down for 10 seconds. Retrieve 10 µL from volumetric centre of PCR tube and dispense into 2nd PCR tube repeating exactly the same procedure for further dilutions.

Performance summary



Standard curve: ***m* = -3.484, Y = 37.487, R^2 = 0.99**
Target amplicon Tm: **74.8ºC ± 0.14 SD (74.64 to 74.96 ºC)**
Limit of detection (LODet): **$7.51x10^1$ cp/µL**
Limit of discrimination (LODis): **$7.51x10^2$ cp/µL**

References

1. Deubelbeiss A, Zahno ML, Zanoni M, Bruegger D, Zanoni R. Real-Time RT-PCR for the Detection of Lyssavirus Species. J Vet Med. 2014;2014:476091.

Revision history

1.0 Original document.