Dengue virus (DENV) detection & quantitation 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) based on previously published oligos for DENV virus screening[1]. Dengue virus are group IV ((+)ssRNA) species of an unassigned order, Flaviviridae family, Flavivirus genus related to the Zika, yellow fever, Japanese encephalitis, and West Nile viruses. It has a genome of 11 kb encoding for three structural proteins: capsid protein (C), membrane protein (M) and envelope protein (E) as well as seven nonstructural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b, NS5). It also includes short non-coding regions on both the 5' and 3' ends.

Oligonucleotide primers

Name	Sequence	bp	%GC	Tm	Hair	HomD	HetD	Amplicon
DENV-F	5′-AAg-gAC-TAg-Agg-TTA-KAg-gAg-ACC-C-3′	25	50	57.6	-0.49	-4.41	-4.77	111
DENV-R	5′-ggC-gYT-CTg-TgC-CTg-gAW-TgA-Tg-3′	23	58.7	61.7	-1.6	- 8.99		

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) ΔQ's given in kcal/mol.

Oligonucleotide primer map in artificial gene (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: DENV1-4 artificial gene sequence derived from 5'UTR partial sequence as available from GenBank accession numbers KT279761, JX024758.

Dual (separate RT and PCR) components and conditions

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

First strand synthesis (RT)

		1x
dH_2O	cf	2.6 µL
10 µM Forward oligo	1.125 µM	2.25 µL
10 µM Reverse oligo	1.125 µM	2.25 µL
10 mM dNTPs	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)

		1x
dH_2O	cf	3.7 µL
Master mix	1 x	5 µL
10 µM Forward oligo	150 nM	0.15 µL
10 µM Reverse oligo	150 nM	0.15 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
60 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 the SCRIPT One-Step RT-qPCR GreenMaster (Cat No. PCR-514S).

qRT-PCR		1x
dH_2O	cf	1.78 µL
10 µM Forward oligo	100 nM	0.1 µL
10 µM Reverse oligo	100 nM	0.1 µL
ROX	0.5 x	0.02 µL
2x SCRIPT mix	1 x	5 µL
Template	< 1 µg	3 µL
		vf: 10 µl

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Run Green Master program in Applied 7500 →

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

Note 3: Data acquisition.

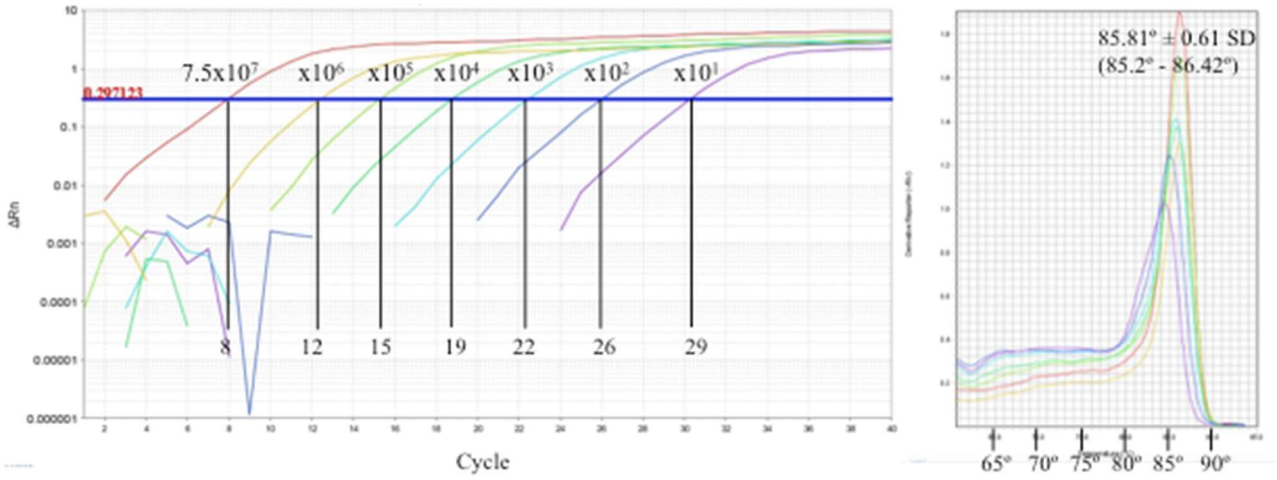
Titration curve preparation for quantitative analysis of viral titres

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 $7.51x10^9$ cp/µL, working dilution of AG prepared at $7.51x10^6$ cp/µL).

		AG stock	dH_20 vol	Ct
Working	$7.5x10^7$ cp/µL	10 µL	90 µL	8.1 ± 0.17
1st	$7.5x10^6$ cp/µL	10 µL	90 µL	12.2 ± 0.25
2nd	$7.5x10^5$ cp/µL	10 µL	90 µL	15.3 ± 0.28
3rd	$7.5x10^4$ cp/µL	10 µL	90 µL	19.2 ± 0.68
4th	$7.5x10^3$ cp/µL	10 µL	90 µL	22.4 ± 0.19
5th	$7.5x10^2$ cp/µL	10 µL	90 µL	26.4 ± 0.42
6th	$7.5x10^1$ cp/µL	10 µL	90 µL	29.4 ± 1.21

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* = -3.57, Y = 36.377, R^2 = 0.996**
Target amplicon Tm: **85.8 ºC ± 0.6 SD (85.2 to 86.42ºC)**
Limit of detection (LODet): **$7.51x10^1$ cp/µL**
Limit of discrimination (LODis): **$7.51x10^1$ cp/µL**

Interpretation

For samples to be considered as positive, their Ct should not be prior to that of the last standard curve's Ct (i.e., < cycles 29, corresponding to the $7.5x10^1$ titre. In addition, the melting curve of the corresponding sample should be higher than surrounding unspecific melting peaks and preferably clean (without accompanying shoulders or unspecific melting peaks).

References

1. Naze F, Le Roux K, Schuffenecker I, Zeller H, Staikowsky F, Grivard P,Michault A, Laurent P. Simultaneous detection and quantitation of Chikungunya, dengue and West Nile viruses by multiplex RT-PCR assays and dengue virus typing using high resolution melting. J Virol Methods. 2009 Dec;162(1-2):1-7. doi:10.1016/j.jviromet.2009.03.006. PubMed PMID: 19773088.

Revision history

1.0 Original document.

2.0 Optimized protocol.

3.0 Changes to document format only.

4.0 Optimized for use un MiniAmp Plus cyclers