Hantavirus (HNT) detection & quantitation by qRT-PCR (EvaGreen).

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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 Sin Nombre Virus (Hantavirus) titres in biological specimens using an one-step SYBR-Green modality (but easily TaqMan adaptable). Sin nombre virus is the type species for North American Hantaviruses while Hantaan Virus is the type species. They are group V ((-)ssRNA) and belong to an unassigned order, Bunyaviridae family, Hantavirus genus. Hantaviruses have a genome comprised of three segments designated small (S, 1696-2083 bp), medium (M, 3613-3707 bp), and large (L, 6530-6550 bp). The S segment encodes the nucleocapsid (N) protein. The M encodes a polyprotein that is cotranslationally cleaved to yield the envelope glycoproteins Gn (formerly G1) and Gc (formerly G2). The L segment encodes the viral transcriptase/replicase.

Oligonucleotide primers

Name	Sequence[1]	bp	%GC	Tm	Hair	HomD	HetD	Size
Hanta-F	5´-ATg-TAT-gTI-AgT-gCW-gAT-gC-3´	20	40%	52.5ºC	0.55	-6.87	-10.1	125
Hanta-R	5´-RYT-RAA-RTg-YTg-YgT-WgT-DgA-YKC-3´	24	42.4%	57.4ºC	1.39	-3.14		
Hanta-R2	5´-gCA-TCA-ACA-ACg-CAA-CAT-TTT-AAC-3´	24	37.5%	54.2ºC	0.23	-4.85		

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. PLEASE NOTE, REVERSE OLIGONUCLEOTIDE PRIMER Hanta-R has been replaced by Hanta-R2 as of October, 2017. This protocol now reflects the optimisations for this new oligonucleotide sequence.

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, Hantavirus specific primer binding sites shown in grey highlight. Hantavirus artificial gene sequence derived from SNV strain Large segment encoding RNA polymerase gene sequence.

One-step qRT-PCR components and conditions (PENDING OPTIMIZATION FOR HANTA-R2)
The One-Step qRT-PCR procedure uses SCRIPT One-Step RT-qPCR GreenMaster (Jena Biosciences Cat No. PCR-514S, 50X ROX reference dye Takara kit RR068A).

qRT-PCR

dH_2O	---	1.8 µL
10 µM oligo mix	100 nM	0.1 µL
50x ROX	0.5 x	0.1 µL
2x SCRIPT	1 x	5 µL
Template	< 1 µg	3 µL
		vf: 10 µl

↓

Run Green Master program in Applied 7500 →

50 ºC	10 min	
95 ºC	5 min	
94 ºC	15 sec	40 cycles
55 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

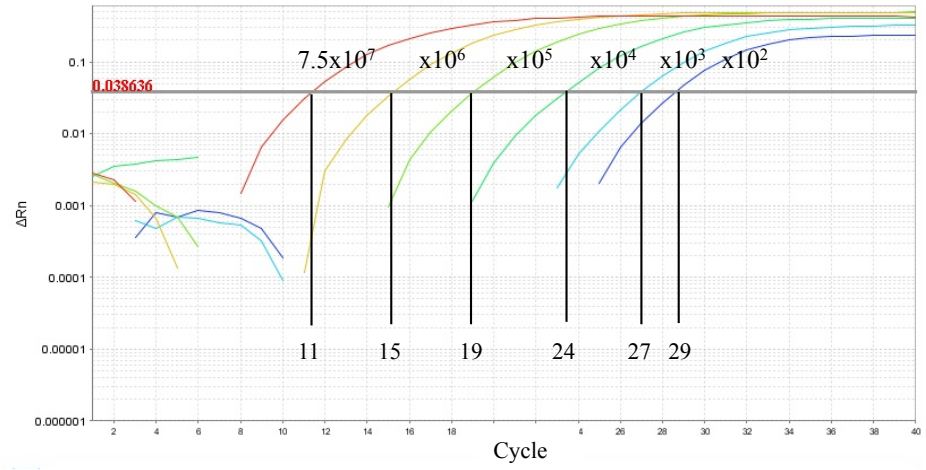
Using Artificial Gene (AG) sequence stock having all viral references (CHIKV, WNV, DENV1-3, DENV2-4, ZIKV, SNV, RABV, TCRV, MERS and SARS) prepare the following log titres (stock currently at $7.51x10^7$ cp/µL, working dilution of AG prepared at $7.51x10^6$ cp/µL).

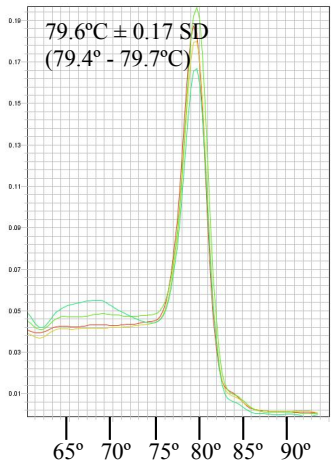
		AG stock	dH_20 vol	Ct mean
1st	$7.5x10^7$ cp/µL	10 µL	90 µL	11.45 (±0.26)
2nd	$7.5x10^6$ cp/µL	10 µL	90 µL	15.40 (±0.3)
3rd	$7.5x10^5$ cp/µL	10 µL	90 µL	19.22 (±0.26)
4th	$7.5x10^4$ cp/µL	10 µL	90 µL	23.55 (±0.33)
5th	$7.5x10^3$ cp/µL	10 µL	90 µL	26.98 (±0.19)
6th	$7.5x10^2$ cp/µL	10 µL	90 µL	28.48 (±0.05)
7th	$7.5x10^1$ cp/µL	10 µL	90 µL	28.71(±0.11)
8th	$7.5x10^0$ cp/µL	10 µL	90 µL	29.81(±0.93)

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 (FOR HANTA-R)





Standard curve: ***m* = -3.55, Y = 40.069, R^2 = 0.984**
Target amplicon Tm: **79.56ºC ± 0.17 SD (79.39 to 79.73 ºC)**
Limit of detection (LODet): **$7.51x10^0$ cp/µL**
Limit of discrimination (LODis): **$7.51x10^3$ cp/µL**

References

1. Mohamed, N., Nilsson, E., Johansson, P., Klingström, J., Evander, M., Ahlm, C., & Bucht, G. (2013). Development and evaluation of a broad reacting SYBR-green based quantitative real-time PCR for the detection of different hantaviruses. *Journal Of Clinical Virology*, *56*(4), 280-285.

Revision history

1.0 Original document.

2.0 Optimized protocol.

3.0 Changes to document format only.