Rabbit calicivirus capsid Taqman RT-qPCR V.1

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Lagovirus biology

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ABSTRACT

To improve the sensitivity and throughput of molecular testing for rabbit caliciviruses we have developed a multiplex TaqMan RT-qPCR assay that detects the three different pathogenic rabbit calicivirus capsid genotypes currently circulating in Australia. Target sequences for all three variants (GI.1c, GI.2, GI.1a) are located within the VP60 capsid sequence. This will also detect subgenomic RNAs, increasing the sensitivity of the method when compared to assays targeting the non-structural gene sequences. Currently this assay is designed to be run as a one-step RT-qPCR assay for high throughput, rapid diagnostic purposes. It has not been designed for quantification of virus loads. For that purpose, the universal lagovirus SYBR-green assay is still recommended (Hall et al, 2018). This assay has not been designed for the detection of recombinant viruses, and will only return the capsid variant/s present in a sample.

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KEYWORDS

rabbit calicivirus; diagnostics; Taqman; multiplex

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GUIDELINES

To avoid contamination, we recommend that a separate area, consumables, and pipettes are used for reaction setup. All reagents should be handled separately to templates.

MATERIALS TEXT

- GI.1c, GI.1a, GI.2 primers and probes - Described in 'Steps'
- SensiFAST Probe no ROX one-step kit Bioline Catalog #BIO-76005
- Neptune fully skirted 96well PCR plate, clear Catalog #3732.x
- Microseal 'B' Adhesive Seals Biorad Sciences Catalog #MSB-1001
- BioRad CFX96 Touch real-time PCR detection system

SAFETY WARNINGS

All work conducted in the laboratory should be undertaken with good laboratory practices in mind. Appropriate personal protective equipment should be worn to protect from biological and chemical hazards.
Disposal of waste produced during RNA extraction should be in accordance with local guidelines.

BEFORE STARTING

Prepare RNA samples for testing. All runs should include a 'no template control' and positive control samples of a GI.1c, GI.1a, and GI.2 RHDV (or a pooled positive control at a minimum).

Plate set-up

1

A	B	C	D	E	F	G	H	I	J	K	L
GI.1	**GI.1**	RNA 6	RNA 6	RNA 15	RNA 15	RNA 24	RNA 24	RNA 33	RNA 33	RNA 41	RNA 41
GI.2	**GI.2**	RNA 7	RNA 7	RNA 16	RNA 16	RNA 25	RNA 25	RNA 34	RNA 34	RNA 42	RNA 42
GI.1a	**GI.1a**	RNA 8	RNA 8	RNA 17	RNA 17	RNA 26	RNA 26	RNA 35	RNA 35	RNA 43	RNA 43
NTC	**NTC**	RNA 9	RNA 9	RNA 18	RNA 18	RNA 27	RNA 27	RNA 36	RNA 36	RNA 44	RNA 44
RNA 1	RNA 1	RNA 10	RNA 10	RNA 19	RNA 19	RNA 28	RNA 28	RNA 37	RNA 37	RNA 45	RNA 45
RNA 2	RNA 2	RNA 11	RNA 11	RNA 20	RNA 20	RNA 29	RNA 29	RNA 38	RNA 38	RNA 46	RNA 46
RNA 3	RNA 3	RNA 12	RNA 12	RNA 21	RNA 21	RNA 30	RNA 30	RNA 39	RNA 39	RNA 47	RNA 47
RNA 4	RNA 4	RNA 13	RNA 13	RNA 22	RNA 22	RNA 31	RNA 31	RNA 40	RNA 40	RNA 48	RNA 48
RNA 5	RNA 5	RNA 14	RNA 14	RNA 23	RNA 23	RNA 32	RNA 32	RNA 41	RNA 41	RNA 49	RNA 49

Plate layout for rabbit calicivirus capsid Taqman RT-qPCR. Samples and controls are routinely run in duplicate. We run individual controls for each strain; at a minimum a pooled positive control should be run.

Reaction set-up

2

- In a dedicated Mastermix hood, UV a 96 well qPCR plate and a Microseal B film for 30 minutes prior to use.
- Thaw reagents during this time.
- Prepare a 10 µM primer mix of GI.1 fwd; GI.1c_rev; GI.2 fwd; GI.2 rv; GI.1a fwd; GI.1a rv. Combine 10 µl of each primer stock (at 100 µM) with 40 µl of nuclease-free water.
- Prepare a Mastermix for number of required reactions + 7% extra to account for pipetting inaccuracies:

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A	B
	µl per reaction
Nuclease-free H2O	5
SensiFAST Probe No-ROX One-Step Mix (2x)	7.5
10µM primer mix	0.6
10µM GI.1c probe	0.15
10µM GI.2 probe	0.15
10µM GI.1a probe	0.15
RiboSafe RNase Inhibitor	0.15
Reverse transcriptase	0.3
Template RNA	1
TOTAL REACTION VOLUME	15 µl

Preparation of Mastermix. Include 7% extra to account for pipetting inaccuracies.

A	B	C
Name	**Sequence (5' - 3')**	**Ref**
GI.1 fwd	TTCAGTTYTGGTAYGCCAATGCTG	This study
GI.1c_rev	AGGCCTGCACAGTCGTAACGTT	Hall et al, 2018
GI.1c probe	FAM - ATGTCGTTCGTACCCTTTAACGGCCCTG - BHQ1	This study
GI.2 fwd	TGGAACTTGGCTTGAGTGTTGA	Duarte et al, 2015
GI.2 rv	ACGAGCGTGCTTGTGGACGG	Duarte et al, 2015
GI.2 probe	HEX - TGTCAGAACTTGTTGAYATYCGCCC - BHQ1	Duarte et al, 2015
GI.1a fwd	AGTGCAAGTTWTKCTGGGAACAACT	This study
GI.1a rv	AAGCCTGYACAGTCGTAGCAGC	This study
GI.1a probe	TexasRed - ATGTCATTCGTGCCYTTTAACAGCCCCA - BHQ2	This study

Primer and probe sets for rabbit calicivirus capsid TaqMan RT-qPCR

- Distribute 14 µl of Mastermix to each well of 96 well PCR plate.
- Add 1 µl of template to respective wells (in duplicate).
  - Ideally, this is done in a dedicated template addition area
- Seal plate with Microseal B film.
- Briefly spin plate down.
- Place plate in qPCR thermocycler (BioRad CFX96) and commence run.

A	B	C
	Temperature (°C)	**Duration**
Reverse transcription	45	10 min
Initial denaturation	95	2 min
40 cycles of:	95	5 sec
	63	20 sec

Thermocycling conditions for rabbit calicivirus capsid TaqMan RT-qPCR

Analysis

3
- Set baseline to 100 RFU for each fluorophore
- Quality control:

1. No amplification in any channel in NTCs.

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2. FAM positive for GI.1 control only (or pooled positive if run).
3. HEX positive for GI.2 control only (or pooled positive if run).
4. TexasRed positive for GI.1a control only (or pooled positive if run).
5. Cq of positive controls within 0.5 Cq of previous runs.
6. Cq standard deviation <0.5 between replicates.

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