TRAINING SCHOOL

ONWARD ratHEV

LABORATORY PROTOCOL SERIES

PROTOCOL 3

Molecular screening for ratHEV

RNA detection and sequencing

COST Action CA24140

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Molecular screening for ratHEV

Two RT-qPCRs are employed in a multiplex format both targeting the beginning 5' of ORF1.

- Kit used: One Step PrimeScript III RT-PCR Kit (Ref RR601B) (Takara Bio, Japan)

The primers, probe, and references are listed in Table 1. Tables 2 and 3 indicate the volume of each reagent used and the thermocycling conditions, respectively. As positive control, we used a ratHEV strain obtained from a specimen of *Rattus norvegicus* (Genbank Accesion N°: OR977609)

Table 1. Primers and probe each qPCR targeting rat hepatitis E virus

ID qPCR	Reference	Target (pb) (position*)	Primers (5'-3')		Probe (5'-3')
			FWD	RVS	
qPCR-1	Sridhar et al. EID, 2018	Met (69) (146-214)	CTTGTTGAGCT YTTCTCCCCT	CTGTACCGG ATGCGACCAA	HEX- TGCAGCTTGTC TTTGARCCC -IABkFQ
qPCR-2	Mulyanto et al. Virus Res 2014	Met (173) (36-208)	CCACGGGGGT TAATACTGC	CGGATGCGA CCAAGAAACAG	FAM-CGGCTACCGCCT TTGCTAATGC-TAMRA

*Genome positions respect to the reference ratHEV sequence under the GenBank Accession number GU345042; Met: methyltransferase

Table 2. Volume of each reagent used for qPCR screening for ratHEV

Reagent	Volume (µl)
Primer FWD qPCR-1 (20µM)	0.75
Primer RVS qPCR-1 (20µM)	0.75
Probe qPCR-1 (20µM)	0.75
Primer FWD qPCR-2 (20µM)	0.75
Primer RVS qPCR-2 (20µM)	0.75
Probe qPCR-2 (20µM)	0.75
Dnase-free water	15.5
PrimeScript III RT-PCR	20
Total Master Mix	*40*
RNA	*10*

Table 3.

Step		Time (sec)	Temperature
UNG Activity		600	25ºC
Retrotranscripción		300	52ºC
Desnaturalización		10	95ºC
Desnaturalización	x45	5	95ºC
Annealing and extension		30	56ºC

Thermocicling conditions for qPCR

Sanger sequencing for partial ratHEV amplification

Several nested PCRs are employed in the lab of GC-26 in IMIBIC to sequence by Sanger the genome of ratHEV. As positive control, we used a ratHEV strain obtained from a specimen of *Rattus norvegicus* (Genbank Accesion N°: OR977609). In the Table 4 you can find the different nested PCRs used and target fragment of the genome. Tables 5 indicates the reagents used for RT-PCR and nested PCR as well as the volumes of each reagent; table 6, the thermocycling conditions.

Table 4. Nested PCRs used for sanger sequencing of rat hepatitis E virus genome

ID qPCR	Reference	Target (pb) (position*)	Primers (5'-3')	
			FWD	RVS
seqPCR Mulyanto	Mulyanto et al. Virus Res 2014	RdRp (880) (4150-5029)	1st PCR: CTTGGTTYAGGGCCATAGAG	1st PCR: CAGCAGCGGCACGAACAGCA
			2nd PCR: TTYAGGGCCATAGAGAAGGC	2nd PCR: ACAGCAAAAGCACGAGCACG
seqPCR Johne	Mulyanto et al. Virus Res 2014	RdRp (333) (4128-4460)	1st PCR: TCGCGCATCACMTTYTTCCARAA	1st PCR: GCCATGTTCCAGACDGTRTTCCA
			2nd PCR: TGTGCTCTGTTTGGCCCNTGGTTYCDG	2nd PCR: CCAGGCTCACCRGARTGYTTCTTCCA
seqPCR A	Caballero Gómez et al. Vet Res 2025	Met (287) (164-450)	1st PCR: TTTGCTAATGCTCAGGTGGT	1st PCR: CATDCCATGAGCACGCAT
			2nd PCR: CCTYTGCAGCTTGTCTTTGA	2nd PCR: GTGCAAAAGGAAAGATCAG
seqPCR E	Caballero Gómez et al. Vet Res 2025	Met (243) (189-450)	1st PCR: CCTYTGCAGCTTGTCTTTGA	1st PCR: CATDCCATGAGCACGCAT
			2nd PCR: CTGTTTCTTGGTCGCATCCG	2nd PCR: GTGCAAAAGGAAAGATCAG

Table 5. Volume of each reagent used for the first and second round of PCR

1ST ROUND		2ND ROUND	
Reagent	Volume (µl)	Reagent	Volume (µl)
Primer FWD (20µM)	0.75	Primer FWD (20µM)	2.5
Primer RVS (20µM)	0.75	Primer RVS (20µM)	2.5
Dnase-free water	18.5	*Dnase-free* water	15
PrimeScript III RT-PCR Kit	20	PCR Master Mix Promega 2X	25
Total Master Mix	*40*	*Total Master Mix*	*40*
RNA	*10*	*DNA product*	*5*

Table 6. Thermocicling conditions for nested PCR

Step		Time (sec)	seqPCR-Mulyanto	seqPCR-Johne	seqPCR-A	seqPCR-E
1st PCR						
UNG Activity		600	25ºC	25ºC	25ºC	25ºC
Reverse transcription		300	52ºC	52ºC	52ºC	52ºC
Denaturation		10	95ºC	95ºC	95ºC	95ºC
Denaturation	x45	5	95ºC	95ºC	95ºC	95ºC
Annealing		30	58ºC	50ºC	55ºC	55ºC
2nd PCR						
Denaturation		120	95ºC	95ºC	95ºC	95ºC
Denaturation	x45	60	95ºC	95ºC	95ºC	95ºC
Annealing		60	58ºC	50ºC	55ºC	55ºC
Extension		60	72ºC	72ºC	72ºC	72ºC
Final extension		300	72ºC	72ºC	72ºC	72ºC

Sanger sequencing for WGS of ratHEV

Recently, a publication coauthored by Dr. Reimar Johne developed a protocol for WGS of different pathogens including ratHEV (doi: 10.1038/s41467-025-60175-9). In that, they designed six primers paired that amplify overlapping regions of ratHEV covering the whole genome of the virus by PCR. This PCR product can be sequenced by NGS following the protocol described in the article. However, it can be also sequenced by Sanger Sequencing, on some occasions*.

The protocol of these PCRs consists on a RT and subsequent PCR of each regions, as follows:

- Retrotranscription
    - Kit used: LunaScript RT SuperMix, New England Biolab (NEB), Ref 3010L
    1. 18 µl are denatured at 65ºC during 5 min. After that, RNA should be placed in ice inmediately at least for 1 minute.
    2. 8 µl of denatured RNA are mixed with 2 µl of Luna RT Supermix
    3. Place this mix in the thermocycler and incubate 2 min 25ºC, 10 min 55ºC, 1 min 95ºC and finally at 4ºC

- PCR
    - Kit used: Q5 Hot Start High-Fidelity DNA Polymerase, NEB, Ref M0493L

Table 7. Primers of each block for Varvamp protocol

ID PCR	Target (pb) (position*)	Primers (5'-3')	
		FWD	RVS
Block 1	1264 (89-1347)	ratHEV1f-FW_0: CTACCGCCTTTGCTAATGCTCAG	ratHEV1r-RW_22 GAVACCCARTCYCKRCACTG
Block 4	1207 (1182-2389)	ratHEV4f-FW_21 SACRCARGGYATATCHATGGG	ratHEV4r-RW_24 ATATDCCRCTHCCRAGNAGGC
Block 2	1600 (2196-3796)	ratHEV2f-FW_27 GCBTTYGAYCGBACYCAGTT	ratHEV2r-RW_31 CACYACYTCYACRTCATCATGCC
Block 5	1413 (3532-4945)	ratHEV5f-FW_33 GTGGCYYTRCAYCAGATGGC	ratHEV5r-RW_46 CAGCAAAAGCACGAGCACGA
Block 3	1233 (4340-5573)	ratHEV3f-FW_42 TGCGGGGYMGRTGGAAGAA	ratHEV3r-RW_55 TCRGTSGARGTGATGGARTTCA
Block 6	1441 (5385-6826)	ratHEV6f-FW_53 GGGTCYAACACGCAYATYATGGC	ratHEV6r-RW_70 YTGACARVGCCTGCACTCG

Table 8. Volume of each reagent is used to amplify each block of Varvamp protocol

Reagent	Volume (µl)
Dnase-free water	17.6
5x Q5 Reaction Buffer	11
5x Q5 High GC Enhancer	11
dNTPs (2,5 mM each)	4.4
Q5 Hot Start High-Fidelity DNA Polymerase	0.55
cDNA	5.5