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## **Standard Operating Procedure**

**for**

**Quantitative detection of norovirus and hepatitis A virus in  
bivalve molluscan shellfish**

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1	2019-11-01	Based on the generic protocol issue 6 by date 05/07/16 produced at the European Union Reference Laboratory for monitoring bacteriological and viral contamination of bivalve molluscs at The Centre for Environment, Fisheries & Aquaculture Science, Weymouth Laboratory

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## 1.0 Introduction

Filter-feeding bivalve molluscan shellfish (BMS) concentrate microbial contaminants occurring in their growing waters and may present a health hazard when consumed raw or lightly cooked. Human enteric viruses including hepatitis A virus (HAV) and norovirus, occurring as a consequence of sewage contamination of growing waters, are the pathogens most frequently associated with illness following shellfish consumption.

A variety of methods for the detection of the low levels of virus found in environmentally contaminated shellfish have been published all of which are reliant upon on the polymerase chain reaction (PCR) for amplification to a detectable threshold. Detection of human enteric viruses in BMS using the PCR is complicated by the complex non-homogenous nature of the sample, by the known presence of amplification inhibitors, and by the low titre of virus present; it is therefore necessary to use a virus/RNA extraction method that produces highly clean RNA preparations that are suitable for PCR detection. Viruses are extracted from the tissues of the BMS digestive glands using treatment with a proteinase K solution. RNA is extracted using a method based on virus capsid disruption with chaotropic reagents followed by adsorption of RNA to silica particles. Real-time RT-PCR monitors amplification throughout the PCR cycle by measuring the excitation of fluorescently labelled molecules. In the 5' fluorogenic nuclease-based real-time RT-PCR assay the fluorescent labels are attached to a sequence-specific nucleotide probe (hydrolysis probe) that also enables simultaneous confirmation of target template. These modifications increase the sensitivity and specificity of the PCR method, and obviate the need for additional amplification product confirmation steps post PCR. Due to the complexity of the method it is necessary to include a comprehensive suite of controls. The method described in this protocol enables quantitative determination of levels of virus RNA in the test sample.

**Note:** The laboratory protocol given here is based on ISO TS 15216-1:2017 with some modifications.

## 2.0 Scope

This procedure describes liberation of HAV and norovirus genogroups I (GI) and II (GII), from BMS tissues and extraction, concentration and quantification of viral RNA. Liberation of viruses is obtained by protease treatment of the BMS tissue. Viral RNA extraction and concentration is by lysis with guanidine thiocyanate and adsorption to silica. Extracted viral RNA is amplified and quantified by real-time RT-PCR. This protocol describes a method for quantitative determination of levels of virus RNA in the test sample and includes production of control materials. The procedure is suitable for live BMS taken directly from production areas or following commercial processing and retail sale. BMS that are commercially sold frozen may also be thawed and tested using this procedure.

## 3.0 Principle

### 3.1 Virus extraction

The BMS digestive glands are removed by a simple dissection and trimming procedure, followed by chopping and protease enzyme treatment to liberate viruses. Following low speed centrifugation the virus-containing supernatant is suitable for viral nucleic acid extraction. Details of addition of a spike process control (mengo virus) to the test samples are also described.

### 3.2 RNA extraction

It is necessary to extract RNA using a method that yields clean RNA preparations to reduce the effect of PCR inhibitors. In this protocol the chaotropic agent guanidine thiocyanate is used to disrupt the viral capsid. RNA is then adsorbed to silica to assist purification through several washing stages. Purified viral RNA is released from the silica into a buffer prior to real-time RT-PCR.

### 3.3 Real-time reverse transcription polymerase chain reaction (real time RT-PCR)

This protocol uses one-step real-time RT-PCR using hydrolysis probes (TaqMan®). In one-step real-time RT-PCR, reverse transcription and PCR amplification are carried out consecutively in the same tube. TaqMan® PCR utilises a short DNA probe with a fluorescent label and a fluorescence quencher attached at opposite ends. The assay chemistry ensures that as the quantity of amplified product increases, the probe is broken down, and the fluorescent signal from the label increases proportionately. Fluorescence may be measured at each stage throughout the cycle. The first point in the PCR cycle at which amplification can be detected for any reaction is proportional to the quantity of template, therefore analysis of the fluorescence plots enables determination of the quantity of target sequence in the sample.

### 4.0 Safety precautions

Standard microbiology safety precautions should be applied throughout. Laboratories should perform a full risk assessment before performing this procedure.

### 5.0 Equipment

- Micropipettes.
- Micropipette tips of a range of sizes, 1000 µl, 200 µl, 20 µl and 10 µl.
- Pipette filler.
- Vortex mixer.
- Shaking incubator operating at 37 °C and 320 rpm or equivalent.
- Thermoshaker operating at 60 °C and 1400 rpm or equivalent.
- Aspirator or equivalent apparatus for removing supernatant.
- Waterbath capable of operating at 60 °C or equivalent.
- Bench centrifuge and rotor capable of running at 3,000 x g with capacity for 15/50ml tubes.
- Microcentrifuge.
- Centrifuge and microcentrifuge tubes/bottles of a range of sizes, 1.5 ml, 15 ml.
- Sterile shucking knife (for opening shellfish) or equivalent.
- Rubber block for shucking (opening) shellfish or equivalent.
- Scissors, scalpels and forceps for dissecting shellfish or equivalent.
- Heavy duty safety glove.
- Sterile Petri dishes.
- Razor blades.
- RNA extraction equipment suitable for extraction using guanidine thiocyanate disruption and silica adsorption-based method.
- If miniMAG/easyMAG extraction system is used; 1.5 ml tubes with screw caps suitable for this system.
- PCR machine with real-time capacity capable of supporting TaqMan® chemistry.
- Consumables for real-time PCR, e.g. optical plates and caps.

## 6.0 Reagents

### 6.1 Reagents used as purchased

- Proteinase K (30 U/mg)
- If NucliSens system is used; magnetic extraction reagents. **BioMerieux**.  
See <http://www.biomerieux.com/>
- If NucliSens system is used; lysis buffer. **BioMerieux**.  
See <http://www.biomerieux.com/>.
- RNA UltraSense™ One-Step Quantitative RT-PCR System. Applied Biosystems™.
- Nuclease free water

### 6.2 Prepared solutions/buffers

- **Proteinase K solution**

Add 20 mg proteinase K (30U/mg) to 200 ml molecular grade water. Shake to dissolve then store in working aliquots at <-15 C for a maximum of 6 months. Once defrosted store aliquots refrigerated and use within 1 week.

- **TaqMan® PCR Buffers**

**Note:** TaqMan® PCR buffers must be prepared no more than 24 hours before use. Short-term storage (<24 hours) at 2-6 °C is appropriate. Always prepare enough buffer for at least one reaction more than required (for larger preparations a greater number of excess reactions may be necessary). With Applied Biosystems real-time machines, Rox should be used at 1 x concentration; for the Stratagene MX3000, Rox can be either used at 0.1 x concentration, or omitted from the Master Mix. For other manufacturers consult the machine instructions.

- **HAV TaqMan® PCR Buffer**

Add the following reagents to a 1.5ml microcentrifuge tube

5µl/reaction	RNA UltraSense 5X Reaction Mix (from RNA UltraSense One-step qRT-PCR system)
1.25µl/reaction	RNA UltraSense Enzyme Mix (from UltraSense system)
0.5µl/reaction or omitted	ROX Reference Dye (1X or 0.1X; see above) (from UltraSense system)
12.5 pmol/reaction	HAV68 (FWD) primer
22.5 pmol/reaction	HAV240 (REV) primer
6.25 pmol/reaction	HAV150 (-) probe (see Appendix 2 for sequences)

Add nuclease free water to a total volume of 20 µl/reaction and mix by vortexing.

- **Norovirus GI TaqMan<sup>®</sup> PCR buffer**

Add the following reagents to a 1.5 ml microcentrifuge tube

5 µl/reaction	RNA UltraSense 5X Reaction Mix (from RNA UltraSense One-step qRT-PCR system)
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1.25 µl/reaction	RNA UltraSense Enzyme Mix (from UltraSense system)
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0.5µl/reaction or omitted	ROX Reference Dye (1X or 0.1X; see above) (from UltraSense system)
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12.5 pmol/reaction	QNIF4 (FWD) primer
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22.5 pmol/reaction	NV1LCR (REV) primer
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6.25 pmol/reaction	TM9 probe (see Appendix 2 for sequences)
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Add nuclease free water to a total volume of 20 µl/reaction and mix by vortexing.

- **Norovirus GII TaqMan<sup>®</sup> PCR buffer**

Add the following reagents to a 1.5 ml microcentrifuge tube

5 µl/reaction	RNA UltraSense 5X Reaction Mix (from RNA UltraSense One-step qRT-PCR system)
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1.25 µl/reaction	RNA UltraSense Enzyme Mix (from UltraSense system)
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0.5µl/reaction or omitted	ROX Reference Dye (1X or 0.1X; see above) (from UltraSense system)
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12.5 pmol/reaction	QNIF2 (FWD) primer
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22.5 pmol/reaction	COG2R (REV) primer
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6.25 pmol/reaction	QNIFS probe (see Appendix 2 for sequences)
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Add nuclease free water to a total volume of 20µl/reaction and mix by vortexing.

- **Mengo virus TaqMan<sup>®</sup> PCR buffer**

Add the following reagents to a 1.5 ml microcentrifuge tube

5 µl/reaction	RNA Ultrasense 5X Reaction Mix (from RNA UltraSense One-step qRT-PCR system)
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1.25 µl/reaction	RNA UltraSense Enzyme Mix (from UltraSense system)
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0.5µl/reaction or omitted	ROX Reference Dye (1X or 0.1X; see above) (from UltraSense system)
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12.5 pmol/reaction	Mengo 110 (FWD) primer
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22.5 pmol/reaction	Mengo 209 (REV) primer
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6.25 pmol/reaction	Mengo 147 probe (see Appendix 2 for sequences)
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Add nuclease free water to a total volume of 20 µl/reaction and mix by vortexing.

### 6.3 Control materials

- **Mengo virus process control material**

**Note:** for preparation of this control material laboratories will require cell culture facilities including incubator(s), preferably with controllable CO<sub>2</sub> levels, cell culture consumables (flasks etc.) and media.

Mengo virus<sup>1</sup> should best be grown in a 5 % CO<sub>2</sub> atmosphere (with open vessels) or an uncontrolled atmosphere (closed vessels) on 80-90 % confluent monolayers of HeLa cells (ATCC CCL-2). Recommended cell culture medium for this cell line is

Eagle's minimum essential medium with 2 mM L-glutamine  
Earle's BSS, adjusted to 1.5 g/l sodium bicarbonate  
0.1 mM non-essential amino acids  
1.0 mM sodium pyruvate  
1 % streptomycin/penicillin  
10 % (growth) or 2 % (maintenance) foetal bovine serum

Alternatively virus can be grown on FRhK-4 cells (ATCC CRL-1688). Recommended cell culture medium for this cell line is

Dulbecco's modified Eagle's medium with 4 mM L-glutamine  
adjusted to 1.5 g/l sodium bicarbonate  
4.5 g/l glucose  
1 % streptomycin/penicillin  
10 % (growth) or 2 % (maintenance) foetal bovine serum

To prepare mengo virus for process control, freeze and thaw a culture flask in which at least 75 % cytopathic effect (CPE) has been reached, centrifuge flask contents at 3000 x g for 10min to clarify and retain supernatant. Dilute by a minimum factor of 10x in sample buffer, e.g. PBS, split into single use aliquots and store frozen at -70 °C. This dilution must allow for inhibition-free detection of the process control virus genome using real-time RT-PCR but still be sufficiently concentrated to allow reproducible determination of the lowest dilution used for the process control virus RNA standard curve.

- **Double-stranded DNA (dsDNA)**

Control plasmids designed by the EURL have been ordered from Eurofins Genomics, <https://www.eurofinsgenomics.eu/en/gene-synthesis-molecular-biology/gene-synthesis/new-gene-order-wizard/>.

Target sites for HAV, norovirus GI and GII have been selected and inserted into pEX-A2 plasmids, containing the ampicillin-resistance gene. Viral sequences surrounding the target sites have been included. To facilitate the production of EC RNA a promotor sequence for T7 RNA polymerase is inserted upstream the viral sequences and downstream the viral sequences is a single cutting restriction site for *HindIII* added. Further, all PCR target sequences have a *BamHI* restriction site inserted to enable the cleavage of PCR products generated from control plasmids.

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<sup>1</sup> Mengo virus strain MC<sub>0</sub> is recommended. It is a genetically modified organism (GMO). MC<sub>0</sub> has the same growth properties as wild type but has an avirulent phenotype. MC<sub>0</sub> can be bought from Spanish Type Culture Collection (CECT) <https://www.uv.es/uvweb/spanish-type-culture-collection/en/news/cect-incorporates-mengovirus-vmco-public-catalogue-1285923246228/Novetat.html?id=1286062092457>

In laboratories where use of GMO is prohibited or problematic, a different process control virus should be used.



A precise description of the plasmids can be found in Appendix 1. The plasmids can be ordered from Eurofins Genomics by describing them accordingly.

For quantitative PT distributions the EURL can supply, *HindIII* cleaved and gel purified, plasmids described in Appendix 1.

Circular plasmids for transformation of E.coli could be distributed on request, but direct ordering from Eurofins Genomics is probably easier and more cost effective.

Control plasmids, as they are described in ISO 15216-1, has been used and distributed by the previous EURL for monitoring bacteriological and viral contamination of bivalve molluscs at the Cefas laboratory. Plasmids distributed by Cefas should give similar quantification results as the plasmids used by the EURL, but deviations can occur.

Plasmids described in ISO 15216-1 was developed by Prof. Albert Bosch (HAV; Costafreda et al., 2006) and Dr. Soizick LeGuyader (norovirus; Le Guyader et al., 2009). For HAV; control plasmid was constructed by ligating the target DNA sequence into the pGEM-3Zf(+) vector (Promega; <https://se.promega.com/-/media/files/resources/protocols/technical-bulletins/0/pgem-3zf-vector-protocol-2.pdf?la=en>) at a *HincII* restriction site such that the target sequence was downstream of a promoter sequence for the SP6 RNA polymerase. Norovirus GI and GII control plasmids were separately constructed by ligating the target DNA sequence into the pGEM-3Zf(+) vector at a *SmaI* restriction site such that in each case the target sequence was downstream of a promoter sequence for the T7 RNA polymerase.

Separate control plasmids for each target virus can also be constructed by individual labs by ligating the target DNA sequence into a suitable plasmid vector such that the target sequence is downstream of a promoter sequence for RNA polymerase and upstream a single cutting restriction site.

**Note:** for preparation of such control materials laboratories will require capabilities for transformation and growth in solid and liquid media of E.coli, capabilities or kits for plasmid preparation, conventional PCR and purification of DNA from reaction mixes (in addition to the listed products) and a spectrophotometer capable of measuring at 260 and 280 nm or a fluorometer for measuring DNA concentration with an appropriate kit.

The plasmids should be transformed and maintained in, and purified from, E. coli cells using standard molecular and microbiology techniques. Following purification of plasmid by e.g. commercial miniprep, linear DNA molecules suitable for use as quantification controls can be generated by linearisation of the plasmid with a single cutting enzyme which does not cut within the target insert. For the plasmids used by the EURL, *HindIII* can be used for all three plasmids. Plasmids described in ISO 15216-1 should be cleaved with *EcoRI* enzyme for norovirus GI and GII and for the HAV plasmid *XbaI* should be used.

Following generation of linear control DNA, a small amount should be cleaned up using e.g. a commercial PCR purification kit.

At the EURL, cleaved plasmids are visualised by gel electrophoresis. Gel bands are excised and subsequently purified with MinElute Gel Extraction Kit (Qiagen). For detailed information on how the EURL produce linear dsDNA see: "Standard operating procedure for Preparation of dsDNA and external control RNA for detection of hepatitis A virus and norovirus GI and GII".

<https://www.livsmedelsverket.se/globalassets/english/production-control-trade/eurl/sop-preparation-of-dsDNA-and-external-control-rna-for-detection-of-hepatitis-a-virus-and-norovirus-gi-and-gii.pdf>

After purification, concentration of DNA can be calculated using spectral absorption at 260 nm (spectral absorption at 280nm should also be carried out to monitor the purity of the DNA preparation, highly pure DNA should have an A260/280 ratio of around 1.8).

Multiplication of the A260 value by  $5 \times 10^{-8}$  (and by any dilution factor involved) will give the concentration of DNA in g/ $\mu$ l.

If fluorometric quantification is used, follow the manufacturer's instructions using the appropriate kit for DNA quantification.

Divide the concentration of DNA by the mass in g of a single dsDNA molecule to calculate the concentration of DNA in copies/ $\mu$ l (the mass of an individual dsDNA molecule may be calculated by multiplying the length in bp by 607.4 (the molecular weight of an average bp) and dividing by the Avogadro constant ( $6.022 \times 10^{23}$ ), e.g. a molecule of 3000bp will have a mass of  $3.02 \times 10^{-18}$ g).

For the (linearised) plasmids used by the EURL the masses are as follows:-

HAV	$3.05 \times 10^{-18}$ g	(3024 bp)
Norovirus GI	$2.74 \times 10^{-18}$ g	(2717 bp)
Norovirus GII	$2.75 \times 10^{-18}$ g	(2725 bp)

For the (linearised) plasmids described in ISO 15216-1 the masses are as follows:-

HAV	$3.41 \times 10^{-18}$ g	(3383 bp)
Norovirus GI	$3.32 \times 10^{-18}$ g	(3287 bp)
Norovirus GII	$3.32 \times 10^{-18}$ g	(3292 bp)

The preparation of linearised dsDNA should then be diluted with a suitable buffer (e.g. TE buffer) to a concentration of approximately  $1 \times 10^4$  -  $1 \times 10^5$  copies/ $\mu$ l, and stored in single use aliquots at 4 °C for up to 24 hrs, at -15 °C or below for up to 6 months, or at -70 °C or below for longer periods.

NOTE: do not use water only to dilute dsDNA to working concentration.

- **External control RNA (EC RNA)**

**Note:** for preparation of these control materials laboratories will require capabilities for transformation and growth in solid and liquid media of *E. coli*, capabilities or kits for plasmid preparation, purification of DNA from reaction mixes (in addition to the listed products) and a spectrophotometer capable of measuring at 260nm or a fluorometer (e.g. Qubit® 3.0 Fluorometer with a Qubit® RNA HS kit, Thermo Fisher Scientific, Waltham, United States).

Linearised dsDNA control plasmids as described above are used for the production of EC RNA.

EC RNA should be transcribed from 100-1000 ng of purified linearised plasmid DNA using an in vitro RNA transcription reaction mix prepared as recommended by the manufacturer of the relevant RNA polymerase enzyme. Following

incubation, digestion of the DNA template using RNase-free DNase should be carried out according to the manufacturer's protocol.

For the plasmids described in ISO 15216-1, EC RNA can be in vitro transcribed using the SP6/T7 Riboprobe combination system (**Promega**, see <http://www.promega.com/>. For information, cat no. P1460) as follows:

1. Add the following components at room temperature in the order listed:

5X transcription buffer	20 µl
100 mM DTT	10 µl
RNasin	2.5 µl
rATP, rGTP, rCTP, rUTP mix (2.5mM each)	20 µl
linearised template DNA (max 0,1-1µg/µl)	concentration dependent
T7 polymerase	3 µl
<b>OR</b>	
SP6 polymerase	3 µl
Nuclease free water	up to 100 µl

Mix by pipetting

2. Incubate for 2 hours at 37 °C.

3. Add 5 µl RQ1 RNase-free DNase to the reaction.

4. Incubate for 15 min at 37 °C.

For the plasmids used at the EURL the same protocol can be used but the Riboprobe® Combination System T3/T7 RNA Polymerase (Promega) is used at the EURL instead of SP6/T7 Riboprobe combination system. For all plasmids the T7 RNA polymerase should be used. Detailed information on how the EURL produce EC RNA, see "Standard operating procedure for Preparation of dsDNA and external control RNA for detection of hepatitis A virus and norovirus GI and GII". <https://www.livsmedelverket.se/globalassets/english/production-control-trade/eurl/sop-preparation-of-dsdna-and-external-control-rna-for-detection-of-hepatitis-a-virus-and-norovirus-gi-and-gii.pdf>

Regardless of the method used for in vitro transcription, the RNA should then be purified using RNA purification reagents (e.g. **QIAGEN** RNeasy Mini Kit, see <http://www1.qiagen.com/>, using the manufacturer's RNA cleanup protocol) and eluting in 50-100 µl RNase-free water.

The RNA preparation should be checked for freedom from significant contamination with DNA by assaying for target both with and without RT activity, for example by assaying with both TaqMan® Master Mix where RT has been deactivated by heating at 95 °C, and untreated Master Mix. If levels of DNA contamination higher than 0.1 % are found, the preparation should be subjected to further treatment(s) with DNase.

The concentration of RNA can then be calculated using spectral absorption at 260 nm or a fluorometer with an appropriate kit for RNA quantification.

Multiplication of the A260 value by 4x10<sup>-8</sup> (and by any dilution factor involved) will give the concentration of RNA in g/µl. For fluorometer, follow the instructions from the manufacturer.

Divide this number by the mass in g of a single EC RNA molecule to calculate the concentration of DNA in copies/µl (the mass of an individual RNA molecule can be calculated by multiplying the RNA length in ribonucleotides by 320.5 (the

molecular weight of an average ribonucleotide) and dividing by the Avogadro constant ( $6.022 \times 10^{23}$ ) e.g. an RNA molecule of 200 ribonucleotides will have a mass of  $1.06 \times 10^{-19}$  g

For the EC RNAs described in ISO 15216-1 the masses are as follows:

HAV	$1.33 \times 10^{-19}$ g	(250 b)
Norovirus GI	$6.73 \times 10^{-20}$ g	(126 b)
Norovirus GII	$7.00 \times 10^{-20}$ g	(131 b)

For EC RNA used at the EURL the masses are as follows:

HAV	$2.92 \times 10^{-19}$ g	(549 b)
Norovirus GI	$1.30 \times 10^{-19}$ g	(245 b)
Norovirus GII	$1.35 \times 10^{-19}$ g	(253 b)

The preparation of RNA transcripts should then be diluted with a suitable buffer (e.g. TE buffer) to a concentration of approximately  $1 \times 10^4$  -  $1 \times 10^5$  transcripts/ $\mu$ l, and frozen in single use aliquots.

**Note:** do not use water only to dilute RNA transcripts to working concentration.

## 7.0 Method

### 7.1 Virus extraction

Immediately before any batch of samples is processed, pool together sufficient aliquots of mengo virus process control material for use with all samples (allow 10  $\mu$ l per sample plus 25  $\mu$ l excess).

Retain a 20  $\mu$ l subsample of pooled material for RNA extraction and preparation of the standard curve. Store at 4 °C for a maximum of 24 hrs or at -20 °C for longer periods.

Select a minimum of 10 live, or frozen, undamaged animals (sufficient shellfish to produce 2 g of digestive glands must be used). Wash off any mud from the shell.

Place the shellfish on a rubber block. Open the shells with a clean knife. Ensure the hand holding the shellfish is protected with a heavy duty safety glove.

Dissect out the digestive glands using scissors and forceps (or equivalent tools).

Transfer to a clean petri dish and trim the digestive gland by removing as much as possible of adipose tissue (fat that is light coloured)

Chop finely with a razor blade.

Transfer a 2 g portion of chopped glands into a centrifuge tube.

If the digestive glands are not processed immediately, store at 4 °C for a maximum of 24 h, at -15 °C or below for up to 6 months, or at -70 °C or below for longer periods (any digestive glands remaining after taking the 2 g portion can be stored at -15 °C or below for up to 6 months, or at -70 °C or below for longer periods.)

Add 10  $\mu$ l of mengo virus process control material directly onto the 2 g portion, then add 2 ml of proteinase K solution and mix well.

Incubate at 37 °C in a shaking incubator or equivalent at 320 rpm for 60 min.

Carry out a secondary proteinase K incubation by placing the tube in a water-bath or equivalent at 60 °C for 15 min.

Centrifuge at 3000 x g for 5 min, decant the supernatant, measure and record the volume and retain for downstream testing (RNA extraction). Process immediately, or store at 4 °C for a maximum of 24 h, at -15 °C or below for up to 6 months, or at -70 °C or below for longer periods.

## 7.2 RNA extraction

**Note:** for every set of samples a negative extraction control (NEC) consisting of 500 µl water should be extracted in parallel unless a negative process control is not included.

**Note:** Below is the NucliSens protocol from bioMérieux described. If an equivalent method is used follow the manufacturer's instructions.

For each test sample, add 2 ml of NucliSens lysis buffer to a tube. Add 500 µl of supernatant produced in 7.1 and mix by vortexing briefly.

In addition, for each batch of mengo virus process control material used with the samples under test, add 2 ml of NucliSens lysis buffer to an additional tube. Add 10 µl of process control material (retained in 7.1) and 500 µl of water and mix by vortexing briefly.

Incubate for 10 min at room temperature.

Add 50 µl of well-mixed magnetic silica solution to the tube and mix by vortexing briefly.

Incubate for 10 min at room temperature.

Centrifuge for 2 min at 1,500 x g then carefully discard supernatant by e.g. aspiration.

Add 400 µl wash buffer 1 and resuspend the pellet by pipetting/vortexing.

Transfer suspension to a 1.5 ml screw-cap tube. Wash for 30 sec using the automated wash steps of the miniMAG/easyMAG extraction systems or by vortexing. After washing allow silica to settle using magnet of the miniMAG/easyMAG extraction system. Discard supernatant by e.g. aspiration.

Separate tubes from magnet, then add 400 µl wash buffer 1. Resuspend pellet, wash for 30 sec, allow silica to settle using magnet then discard supernatant.

Separate tubes from magnet, then add 500 µl wash buffer 2. Resuspend pellet, wash for 30 sec, allow silica to settle using magnet then discard supernatant. Repeat.

Separate tubes from magnet, then add 500 µl wash buffer 3. Wash for 15 sec, allow silica to settle using magnet then discard supernatant.

**Note:** samples should not be left in wash buffer 3 for longer than strictly necessary

Add 100 µl elution buffer. Cap tubes and transfer to thermoshaker or equivalent.

Incubate for 5 min at 60 °C with shaking at 1400 rpm.

Place tubes in magnetic rack and allow silica to settle, then transfer eluate to a clean tube and retain for TaqMan® analysis. Process immediately, or store at 4 °C for a maximum of 24 h, at -15 °C or below for up to 6 months, or at -70 °C or below for longer periods.

## 7.3 TaqMan® analysis – general requirements

**Note:** for certain applications more or fewer TaqMan® replicates can be used for the sample or various control RNAs. Where variations are made with regards to this method this approach should be subject to verification by the user laboratory. In addition results generated using 10<sup>-1</sup> sample RNA are used only in the event that RT-PCR inhibition is >75 % for undiluted sample RNA; it is therefore permitted for laboratories to omit 10<sup>-1</sup> sample RNA from the initial analysis of target virus and process control virus. In this case, where RT-PCR

inhibition is >75 % for undiluted sample RNA, real-time RT-PCR analysis for any affected target viruses and for the process control virus shall be repeated using  $10^{-1}$  sample RNA.

TaqMan<sup>®</sup> analysis for all targets need not be carried out on the same plate – however the following restrictions must be observed;

Full sets of target assay control reactions (dsDNA dilution series, EC RNA and water only) should be used for every plate where sample RNA is assayed for that target.

Full sets of mengo virus assay control reactions (RNA dilution series from all relevant batches of mengo virus process control material and water only) must be included on every plate where sample RNA is assayed for mengo virus.

Prepare TaqMan<sup>®</sup> Master Mixes immediately before starting procedure.

#### **7.4 TaqMan<sup>®</sup> plate set-up - analysis of target viruses**

**Note:** this section describes plate set-up for a single target virus.

Before starting 96-well real-time PCR plate preparation, prepare  $10^{-1}$  dilutions of each sample RNA in nuclease free water.

Dependent on the target assays to be used, prepare  $10^{-1}$ ,  $10^{-2}$ ,  $10^{-3}$  and  $10^{-4}$  dilutions of target dsDNA in a suitable buffer (e.g. TE buffer).

For each sample and each target assay add 5 µl of undiluted and  $10^{-1}$  sample RNA to three wells of the plate each.

For each negative extraction control and each target assay add 5 µl of undiluted RNA to two wells.

For each target assay add 5 µl of nuclease-free water to three wells.

For each target assay add 5 µl of undiluted,  $10^{-1}$ ,  $10^{-2}$ ,  $10^{-3}$  and  $10^{-4}$  dsDNA to two wells each.

For each target assay add 1 µl of undiluted EC RNA to one well for each undiluted sample RNA, one well for each  $10^{-1}$  sample RNA and one well containing water only.

Add 20 µl of the relevant TaqMan<sup>®</sup> Master Mix to each well.

#### **7.5 TaqMan<sup>®</sup> plate set-up - analysis of mengo virus**

For each batch of mengo virus process control material extracted (7.2) prepare  $10^{-1}$ ,  $10^{-2}$  and  $10^{-3}$  dilutions of mengo virus RNA in a suitable buffer (e.g. TE buffer).

Add 5 µl of undiluted and  $10^{-1}$  sample RNA to one well of the plate each.

For each negative extraction control add 5 µl of undiluted RNA to one well.

For each batch of mengo virus process control add 5 µl of undiluted,  $10^{-1}$ ,  $10^{-2}$  and  $10^{-3}$  mengo virus RNA to one well each.

Add 5 µl of nuclease-free water to one well.

Add 20 µl of the mengo virus TaqMan<sup>®</sup> Master Mix to each well.

See layout on following page for example TaqMan<sup>®</sup> plate testing one sample for all four targets.

SOP - Quantitative detection of norovirus and hepatitis A virus in bivalve molluscan shellfish. Version: 1

Example plate layout (single sample – all assays on one plate) NOTE: This layout includes wells with 10<sup>-1</sup> sample RNA, however these can be omitted from the initial analysis as described in 7.3 (see below)

Test sample (undiluted)	Test sample (undiluted)	Test sample (-1)	Test sample (-1)	Test sample (undiluted) + HAV EC RNA	Test sample (-1) + HAV EC RNA	H <sub>2</sub> O + HAV EC RNA	Negative extraction control	Negative extraction control	H <sub>2</sub> O	H <sub>2</sub> O	
HAV dsDNA (undiluted)	HAV dsDNA (undiluted)	HAV dsDNA (-1)	HAV dsDNA (-1)	HAV dsDNA (-2)	HAV dsDNA (-2)	HAV dsDNA (-3)	HAV dsDNA (-3)	HAV dsDNA (-4)	HAV dsDNA (-4)		
Test sample (undiluted)	Test sample (undiluted)	Test sample (-1)	Test sample (-1)	Test sample (undiluted) + GI EC RNA	Test sample (-1) + GI EC RNA	H <sub>2</sub> O + GI EC RNA	Negative extraction control	Negative extraction control	H <sub>2</sub> O	H <sub>2</sub> O	
GI dsDNA (undiluted)	GI dsDNA (undiluted)	GI dsDNA (-1)	GI dsDNA (-1)	GI dsDNA (-2)	GI dsDNA (-2)	GI dsDNA (-3)	GI dsDNA (-3)	GI dsDNA (-4)	GI dsDNA (-4)		
Test sample (undiluted)	Test sample (undiluted)	Test sample (-1)	Test sample (-1)	Test sample (undiluted) + GII EC RNA	Test sample (-1) + GII EC RNA	H <sub>2</sub> O + GII EC RNA	Negative extraction control	Negative extraction control	H <sub>2</sub> O	H <sub>2</sub> O	
GII dsDNA (undiluted)	GII dsDNA (undiluted)	GII dsDNA (-1)	GII dsDNA (-1)	GII dsDNA (-2)	GII dsDNA (-2)	GII dsDNA (-3)	GII dsDNA (-3)	GII dsDNA (-4)	GII dsDNA (-4)		
Test sample (undiluted)	Test sample (-1)	Process control virus RNA (undiluted)	Process control virus RNA (-1)	Process control virus RNA (-2)	Process control virus RNA (-3)	Negative extraction control	H <sub>2</sub> O				

HAV assay
Norovirus GI assay
Norovirus GII assay
Mengo virus assay

5 µl RNA (+/- 1 µl EC RNA) & 20 µl Master Mix per well

### 7.6 TaqMan® assay run parameters

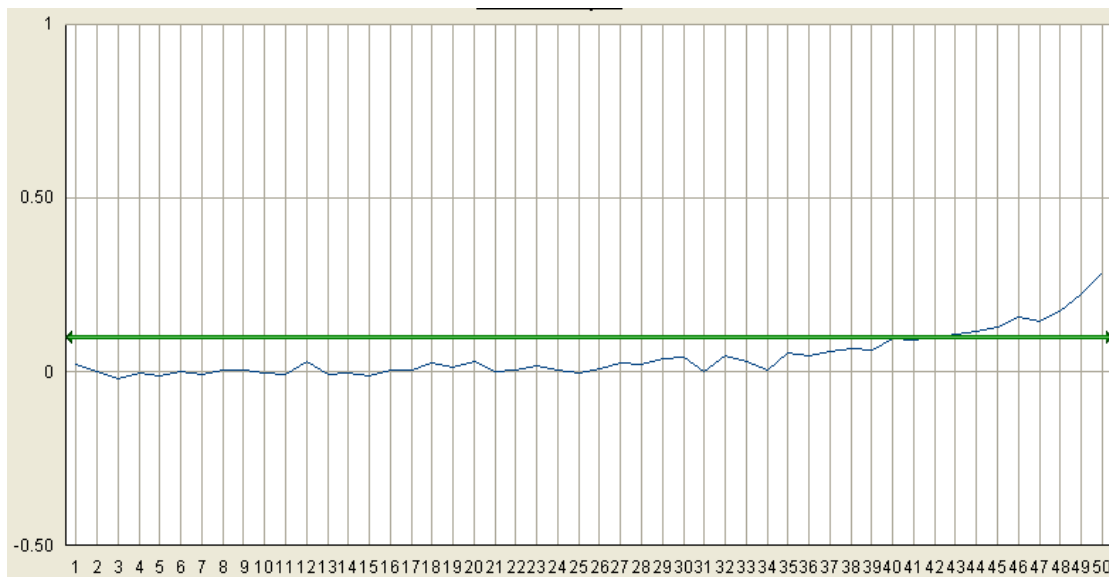
Run the TaqMan® assay with the following parameters:-

Step description	Temperature and time	Number of cycles	
RT	55 °C for 1 h	1	
Preheating	95 °C for 5 min	1	
Amplification	Denaturation	95 °C for 15 s	
	Annealing-	60 °C for 1 min	45
	extension	65 °C for 1 min	

### 7.7 Analysis of results

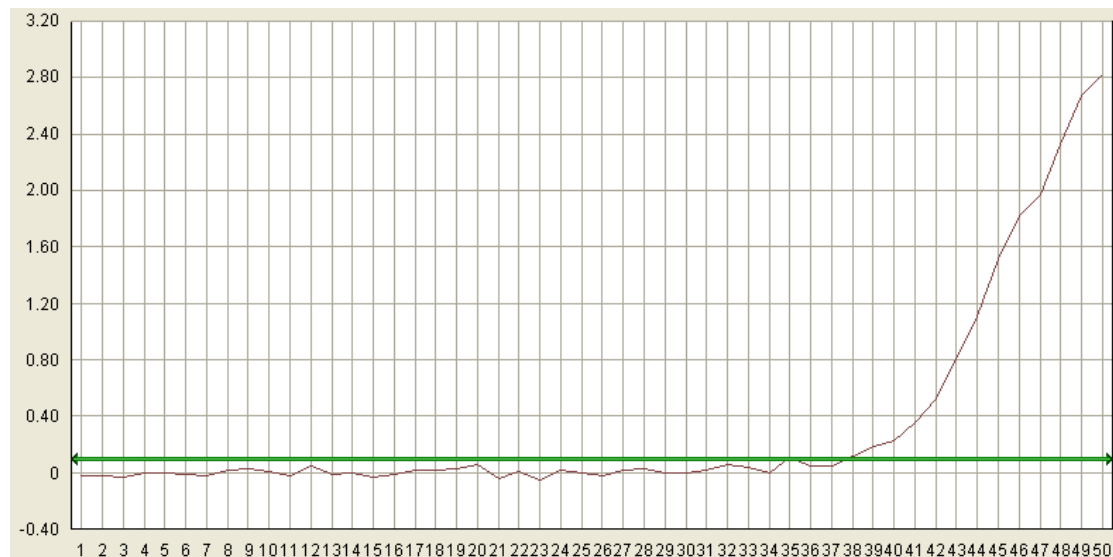
Analyse the amplification plots using the approach recommended by the manufacturer of the real-time PCR machine. The threshold should ideally be set so that it crosses the area where the amplification plots (logarithmic view) are parallel (the exponential phase). Alternatively, thresholds are set automatically by the software. Some platforms do not have logarithmic view, still threshold should be set early in the exponential phase.

Check all amplification plots to identify false positive results caused by high or uneven background signal. Results for any wells affected in this way should be regarded as negative e.g.





Check all amplification plots to identify true positive plots where the recorded Cq value is significantly distorted by high or uneven background signal. Approximate correct Cq values should be noted (in addition to the recorded value) for any wells affected in this way. Corrected Cq values should be used for all quantity calculations.



For example in this case the recorded Ct value was 34.92, however it should be noted by the participating laboratory that the correct figure should be e.g. 38.

Use the Cq values of each dilution series to create standard curves for each control by plotting the Cq values obtained against log<sub>10</sub> concentration (e.g. log<sub>10</sub> copies per microliter target dsDNA) to determine r<sup>2</sup>, slope and intercept parameters. Do not average Cq values from duplicate reactions prior to plotting.

Curves with r<sup>2</sup> values of <0.980, or where the slope is not between -3.10 and -3.60 (corresponding to amplification efficiencies of ~90-110 %), should not be used for calculations. In these cases, check the Cq values of the standard curve for any outlying values and remove these from the series. No more than two such outlying Cq values shall be removed per series and values from a minimum of three (mengovirus RNA) or four dilutions (dsDNA) must be retained.

Repeat the calculations to determine r<sup>2</sup>, slope and intercept parameters. Where the modified curve has an r<sup>2</sup> value of <0.980, or where the slope is not between -3.10 and -3.60, the modified slope shall not be used for calculations.

**Note:** subject to the existence of supporting data, laboratories can apply more stringent acceptance criteria for r<sup>2</sup> (e.g. values <0.990 not permitted) or slope parameters

Use the Cq value for the undiluted sample RNA + EC RNA well to determine the RT-PCR inhibition levels for each sample and each target by reference to the Cq value of the water + EC RNA well and the slope of the dsDNA standard curve as follows:-

$$\text{RT-PCR inhibition} = (1 - 10^{(\Delta Cq/m)}) \times 100 \%$$

where  $\Delta Cq = Cq \text{ value [sample RNA + EC RNA]} - Cq \text{ value [water + EC RNA]}$

and where m = slope of the dsDNA standard curve

A sample (+ EC RNA) producing the same Cq values as undiluted EC RNA will have an RT-PCR inhibition level of 0 %.

If the RT-PCR inhibition level is <75 % results for the undiluted RNA should be used for that sample and target. If the RT-PCR inhibition level is >75 % repeat calculation with the 10<sup>-1</sup> sample RNA + EC RNA wells for the same target. If the RT-PCR inhibition level using the 10<sup>-1</sup> RNA is <75%, results for the 10<sup>-1</sup> RNA should be used for that sample and target. If RT-PCR inhibition levels for both undiluted and 10<sup>-1</sup> sample RNA are >75% results are not valid and the sample should be retested.

Use the Cq value for the mengo virus assay from the test sample RNA well (undiluted or 10<sup>-1</sup> dependent on the RT-PCR inhibition results; see above) to estimate mengo virus recovery by reference to the mengo virus RNA standard curve as follows (if 10<sup>-1</sup> sample RNA results are used multiply by 10 to correct for the dilution factor):

$$\text{Mengo virus recovery} = 10^{(\Delta Cq/m)} \times 100 \%$$

where  $\Delta Cq = Cq \text{ value [sample RNA]} - Cq \text{ value [undiluted process control virus RNA]}$

and where  $m = \text{slope of the process control virus RNA standard curve}$

A sample producing the same Cq value as undiluted mengo virus RNA will have a mengo virus recovery of 100 %. To determine the extraction efficiency divide the mengo virus recovery by 0.5 and multiply by the total measured volume of supernatant (7.1). Where the extraction efficiency is <1 % sample results are not valid and the sample should be retested.

For each sample and target virus, take the Cq values for the sample RNA only wells (undiluted or 10<sup>-1</sup> dependent on the RT-PCR inhibition results; see above) and use these to calculate target concentrations (in detectable virus genome copies/ $\mu\text{l}$  RNA) for each replicate by reference to the relevant dsDNA standard curve as follows:-

$$\text{concentration} = 10^{(\Delta Cq/m)}$$

where  $\Delta Cq = Cq \text{ value [sample RNA]} - \text{standard curve intercept}$

and where  $m = \text{slope of the dsDNA standard curve}$

Negative replicates should be given a concentration of zero copies/ $\mu\text{l}$  RNA. For each sample calculate the average of the concentrations for both replicates.

Multiply this value by 200 (undiluted RNA) or 2 000 (10<sup>-1</sup> RNA) then multiply by the total volume of supernatant in ml (7.1) to calculate the number of detectable virus genome copies in the entire sample.

To obtain the concentration of target virus in detectable virus genome copies per g divide the number of genome copies in the entire sample by the starting weight (normally 2 g) of the sample.

## 8.0 Uncertainty of test results

Uncertainty inherent in any test method, i.e. instruments, media, analyst performance etc. can be assessed by the repeatability and reproducibility of test results. These should be monitored through control tests analysed alongside sample tests, in-house comparability testing between analysts and external intercomparison exercises, which would highlight any uncertainties within the test methods.

## 9.0 References

- Costafreda MI, Bosch A, Pintó RM. 2006. Development, evaluation, and standardization of a real-time TaqMan reverse transcription-PCR assay for quantification of hepatitis A virus in clinical and shellfish samples. *Appl Environ Microbiol.* 72(6):3846-3855.
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- Hoehne M, Schreier E. 2006. Detection of Norovirus genogroup I and II by multiplex real-time RT-PCR using a 3'-minor groove binder-DNA probe. *BMC Infect Dis.* 6:69.
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- Loisy F, Atmar RL, Guillon P, Le Cann P, Pommepuy M, Le Guyader FS. 2005. Real-time RT-PCR for norovirus screening in shellfish. *J Virol Methods.* 123(1):1-7.
- Maguire AJ, Green J, Brown DWG, Desselberger U, Gray JJ. 1999. Molecular Epidemiology of Outbreaks of Gastroenteritis Associated with Small Round-Structured Viruses in East Anglia, United Kingdom, During the 1996–1997 Season. *J Clin Microbiol.* 37(1): 81-89.
- Pintó RM, Costafreda MI, Bosch A. 2009. Risk assessment in shellfish-borne outbreaks of hepatitis A. *Appl Environ Microbiol.* 75(23): 7350-7355.

## 10.0 Appendix 1. Plasmids used at the EURL

Plasmids were purchased from Eurofins Genomics, with the following insert sequences:

### Hepatitis A virus

pEXA2-T7HAV(1-542)HindIII

Vector: pEX-A2, Eurofins

[https://www.eurofinsgenomics.eu/media/892663/pex-a2\\_map\\_seq\\_v12.pdf](https://www.eurofinsgenomics.eu/media/892663/pex-a2_map_seq_v12.pdf)

#### Insert sequence

```
TAATACGACTCACTATAGGTTCAAGAGGGGTCTCCGGGAATTTCCGGAGTCCCTCTTGGAAGTC
CATGGTGAGGGGACTTGATAACCTCACCGCCGTTTGCCTAGGCTATAGGCTAAATTTCCCTTTC
CCTTTTGGATCCCTTTCCTATTCCTGGATCCTTGTTTTGCTTGTAATATTAATTCCTGCAGG
TTCAGGGTTCTTAAATCTGTTTCTCTATAAGAACAATCATTTTTACGCTTCTGTCTTCTTTC
TTCCAGGGCTCTCCCTTGCCCTAGGCTCTGGCCGTTGCGCCCGGCGGGGTCAACTCCATGATT
AGCATGGAGCTGTAGGAGTCTAAATTGGGGACACAGATGTTTGGAACGTCACCTTGCAGTGTTA
ACTTGGCTTTTCATGAATCTCTTTGATCTTCCACAAGGGGTAGGCTACGGGTGAAACCTCTTAGG
ATCCGGCTAATACTTCTATGAAGAGATGCCTTGGATAGGGTAACAGCGCGGATATTGGTGAGT
TGTTAAGACAAAAACCATTC AACGCCGGAGGACTGACTCTCATCCAGTGGATGAAGCTTATT
```

Plasmid size: 3024 bp

Transcript size: 549 b

Position: base 1-542 in NC001489.1

Sense: plus

T7 ->

GGAT: extra bases to generate a *Bam*HI-site

ATGC: primers and probe

AAGCTT: Restriction site for *Hind*III

#### PCR-system:

HAV68:	TCACCGCCGTTTGCCTAG
HAV150:	CCTGAACCTGCAGGAATTAA
reverse complement:	TTAATTCCTGCAGGTTTCAGG
HAV240:	GGAGAGCCCTGGAAGAAAG
reverse complement:	CTTCTTCCAGGGCTCTCC

## Norovirus GI

pEXA2-T7GI(128-267)HindIII

Vector: pEX-A2, Eurofins

[https://www.eurofinsgenomics.eu/media/892663/pex-a2\\_map\\_seq\\_v12.pdf](https://www.eurofinsgenomics.eu/media/892663/pex-a2_map_seq_v12.pdf)

### Insert sequence

TAATACGACTCACTATAGG GATTTCAGCAAGGTCATACATGAAATCAAGACTGGTGGATTGGA  
AATGTATGTCCCAGGATGGCAGGCCATGTTCCGCTGGATGCGCTTCCATGACCTCGGATTGTGG  
ACAGGAGATCGCGATCTTCTGC GGATCCGAATTCGTAATGATGATGGCGTCTAAGGACGCTAC  
ATCAAGCGTGGATGGCGCTAGTGGCGCTGGTCAGTTGGTACCGGAGGTTAATGCTTCTGACCCT  
CTTGC AAGCTT

Plasmid size: 2717 bp

Transcript size: 245 bp

Position: base 128-267 in Norwalk M87661

Sense: plus

T7→

GGAT: extra bases to generate a *Bam*HI-site

ATGC: primers and probe

AAGCTT: Restriction site for *Hind*III

### PCR-system:

QNIF4 (F): CGCTGGATGCGNTTCCAT

TM9 (P): TGGACAGGAGATCGC

NV1LCR (R): GTAAATGATGATGGCGTCTAAGG

reverse complement: CCTTAGACGCCATCATCATTAC

## Norovirus GII

pEXA2-T7GII(4935-5180)HindIII

Vector: pEX-A2, Eurofins

[https://www.eurofinsgenomics.eu/media/892663/pex-a2\\_map\\_seq\\_v12.pdf](https://www.eurofinsgenomics.eu/media/892663/pex-a2_map_seq_v12.pdf)

### Insert sequence

TAATACGACTCACTATAGGACAGCAAATTAGCAAGCTAGTCATTGCAGAACTGAAGGAAGGTG  
GCATGGATTTTTACGTGCCAGACAAGAGCCAATGTTTCAGATGGATGAGATTCTCAGATCTGAG  
CACGTGGGAGGGCGATCGCAATCTGGCTCGGATCCAGCTTGTGAATGAAGATGGCGTCGAATG  
ACGCCAACCCATCTGATGGGTCCGCAGCCAACCTCGTCCCAGAGGTCAATAATGAGGTTATGGC  
TCTGGAGCCCGTTAAGCTT

Plasmid size: 2725 bp

Transcript size: 253 bp

Position: base 4935-5180 in Lordsdale (X86557.1)

Sense: plus

T7→

GGAT: extra bases to generate a *Bam*HI-site

ATGC: primers and probe

AAGCTT: Restriction site for *Hind*III

### PCR-system:

QNIF2: ATGTTTCAGRTGGATGAGRTTCTCWGA

QNIFS: AGCACGTGGGAGGGCGATCG

COG2R: TGTGAATGAAGATGGCGTCGA

reverse complement: TCGACGCCATCTTCATTCACA

## 11.0 Appendix 2: Primer and probe sequences

### Norovirus GI

QNIF4 (FW): CGC TGG ATG CGN TTC CAT [da Silva et al., 2007]

NV1LCR (REV): CCT TAG ACG CCA TCA TCA TTT AC [Svraka et al., 2007]

**During the development of ISO 15216, two different probes for norovirus GI were used. Either can be used with the FW and REV primers detailed here.**

NVGG1p (PROBE): TGG ACA GGA GAY CGC RAT CT [Svraka et al., 2007]

Probe labelled 5' 6-carboxyfluorescein (FAM), 3' 6-carboxy-tetramethylrhodamine (TAMRA)

TM9 (PROBE): TGG ACA GGA GAT CGC [Hoehne & Schreier, 2006]

Probe labelled 5' FAM, 3' MGBNFQ (minor groove binder/non-fluorescent quencher)

### Norovirus GII

QNIF2 (FW): ATG TTC AGR TGG ATG AGR TTC TCW GA [Loisy et al., 2005]

COG2R (REV): TCG ACG CCA TCT TCA TTC ACA [Kageyama et al., 2003]

QNIFS (PROBE): AGC ACG TGG GAG GGC GAT CG [Loisy et al., 2005]

Probe labelled 5' FAM, 3' TAMRA

### Hepatitis A virus

HAV68 (FW): TCA CCG CCG TTT GCC TAG [Costafreda et al., 2006]

HAV240 (REV): GGA GAG CCC TGG AAG AAA G [Costafreda et al., 2006]

HAV150(-) (PROBE): CCT GAA CCT GCA GGA ATT AA [Costafreda et al., 2006]

Probe labelled 5' FAM, 3' MGBNFQ

### Mengo virus

Mengo 110 (FW): GCG GGT CCT GCC GAA AGT [Pinto et al., 2009]

Mengo 209 (REV): GAA GTA ACA TAT AGA CAG ACG CAC AC [Pinto et al., 2009]

Mengo 147 (PROBE): ATC ACA TTA CTG GCC GAA GC [Pinto et al., 2009]

Probe labelled 5' FAM, 3' MGBNFQ