National Reference Laboratory for foodborne viruses

Generic Protocol - Quantification of norovirus and hepatitis A virus in bivalve molluscan shellfish (based on ISO 15216-1)

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History of Procedure

Issue	Date	Section	Changes
1	08.03.19		Issue 1
2	30.04.26	Throughout	Minor corrections and improvements to text for clarity
		Throughout	Hyperlinks updated
		Throughout	Catalogue numbers for commercial reagents updated
		6.2	Recipes for Tris solution and EDTA solution added
		7.2	Method for RNA extraction corrected so that 500 µl of supernatant is used rather than the entire sample

1.0 Introduction

Norovirus (NoV) and hepatitis A virus (HAV) are important agents of food-borne human viral illness. One of the food types associated with transmission of norovirus and HAV is bivalve molluscan shellfish (BMS). No routine methods exist to culture these viruses from food matrices. Detection is therefore reliant on molecular methods using the reverse-transcriptase polymerase chain reaction (RT-PCR). As many food matrices contain substances that are inhibitory to RT-PCR, it is necessary to use a virus/RNA extraction method that produces highly clean RNA preparations that are fit-for-purpose. 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. Real-time RT-PCR monitors amplification throughout the PCR cycle by measuring the excitation of fluorescently labelled molecules. In the 5' fluorogenic nuclease real-time RT-PCR assay the fluorescent labels are attached to a sequence-specific nucleotide probe (hydrolysis probe) that enables simultaneous confirmation and quantification 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 quantification of levels of virus RNA in the test sample.

2.0 Scope

This procedure describes liberation, concentration and quantification of NoV genogroups I (GI) and II (GII) and HAV, from BMS tissues. Viral RNA extraction is by lysis with guanidine thiocyanate and adsorption to silica. Extracted viral RNA is amplified and detected by real-time RT-PCR. This protocol describes a method for quantification of virus RNA in the test sample. 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 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.
- Pipettes of a range of sizes, 25 ml, 10 ml, 5 ml.
- Vortex mixer.
- Shaking incubator operating at 37ºC and 320 rpm or equivalent.
- Thermoshaker operating at 60ºC 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 15/50 ml tubes.
- Microcentrifuge.
- Centrifuge and microcentrifuge tubes/bottles of a range of sizes, 1.5 ml, 15 ml, 50 ml etc.
- Sterile shucking knife (for opening shellfish) or equivalent.
- Rubber block for shucking (opening) shellfish or equivalent.
- Scissors and forceps for dissecting shellfish or equivalent.
- Heavy duty safety glove.
- Sterile Petri dishes.
- Razor blades.

- Magnetic rack for 1.5 ml tubes.
- NucliSens miniMAG instrument. **BioMerieux.** NOTE: this item is no longer commercially available – versions of the protocol both with and without this item are presented below.
- [For methods using the miniMAG instrument] 1.5 ml tubes with screw caps.
- 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)
- Tris base
- Ethylenediaminetetraacetic acid (EDTA) disodium dihydrate
- NucliSens magnetic extraction reagents. **BioMerieux**. See http://www.biomerieux.com/ for information. Cat numbers; 280130, 280131, 280132, 280133 etc.
- NucliSens lysis buffer. **BioMerieux**. See http://www.biomerieux.com/ for information. Cat numbers; 280134 etc.
- RNA Ultrasense One-step qRT-PCR system. Thermo Fisher Scientific. See https://www.thermofisher.com/uk/en/home.html for information. Cat number; 11732927.
- Nuclease free water

6.2 Prepared solutions/buffers

Note: Taqman® PCR buffers must be prepared immediately before use. Always prepare enough buffer for at least one reaction more than required (for larger preparations a greater number of excess reactions may be necessary). For the Stratagene MX3000, Rox can be either used at 0.1 x concentration, or omitted from the mastermix. For other manufacturers consult the machine instructions.

- **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 <-15ºC for a maximum of 6 months. Once defrosted store aliquots refrigerated and use within 1 week.

- **Tris solution (1 M)**

Add 12.1 g Tris base and 100 ml molecular grade water to a bottle. Mix with stirring until the solids are dissolved. Adjust the pH to 8.0. Sterilise by autoclaving. Store at 2-6 °C for up to 6 months.

- **EDTA solution (0.5 M)**

Add 18.6 g EDTA disodium dihydrate and 100 ml molecular grade water to a bottle. Mix with stirring until the solids are dissolved. Adjust the pH to 8.0. Sterilise by autoclaving. Store at 2-6 °C for up to 6 months.

- **Tris EDTA (TE) buffer**

Add 1 ml Tris solution, 200 µl EDTA solution and 100 ml molecular grade water to a bottle. Shake to mix. Store at 2-6 °C for up to 6 months. Alternatively use TE buffer from a commercial source.

- **Norovirus GI Taqman® 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)
1.25 µl/reaction	RNA Ultrasense Enzyme Mix (from Ultrasense system)
0.5 µl/reaction	ROX Reference Dye (at recommended concentration; see above) (from Ultrasense system)
12.5 pmol/reaction	QNIF4 (FWD) primer
22.5 pmol/reaction	NV1LCR (REV) primer
6.25 pmol/reaction	NVGG1p or TM9 probe (see Appendix 1 for sequences)

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

- **Norovirus GII Taqman® 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)
1.25 µl/reaction	RNA Ultrasense Enzyme Mix (from Ultrasense system)
0.5 µl/reaction	ROX Reference Dye (at recommended concentration; see above) (from Ultrasense system)
25 pmol/reaction	QNIF2 (FWD) primer
45 pmol/reaction	COG2R (REV) primer
12.5 pmol/reaction	QNIFS probe (see Appendix 1 for sequences)

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

- **Hepatitis A virus Taqman® 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)
1.25 µl/reaction	RNA Ultrasense Enzyme Mix (from Ultrasense system)
0.5 µl/reaction	ROX Reference Dye (at recommended concentration; 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 1 for sequences)

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

- **Mengo virus Taqman® 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)
1.25 µl/reaction	RNA Ultrasense Enzyme Mix (from Ultrasense system)
0.5 µl/reaction	ROX Reference Dye (at recommended concentration; see above) (from Ultrasense system)
12.5 pmol/reaction	Mengo 110 (FWD) primer
22.5 pmol/reaction	Mengo 209 (REV) primer
6.25 pmol/reaction	Mengo 147 probe (see Appendix 1 for sequences)

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_2 levels, cell culture consumables (flasks etc.) and media.

Mengo virus strain MC_0 (Spanish Type Culture Collection - CECT 100000) should be used unless proscribed by e.g. GMO regulations. Mengo virus should best be grown in a 5% CO_2 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 10 min 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 -15 °C or below. 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)**

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, conventional PCR and purification of DNA from reaction mixes (in addition to the listed products) and a spectrophotometer capable of measuring at 260 and 280nm.

Control plasmids used by the UK NRL were 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; pGEM®-3Zf(+/–) Vectors) at a *Hin*cII restriction site such that the target sequence was downstream of a promoter sequence for the SP6 RNA polymerase. For norovirus GI and GII control plasmids were separately constructed by ligating the target DNA sequence into the pGEM-3Zf(+) vector at a *Sma*I restriction site such that in each case the target sequence was downstream of a promoter sequence for the T7 RNA polymerase. The UK NRL may be able to supply these plasmids to UK Official Laboratories upon request.

Alternatively, separate control plasmids for each target virus can 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.

The plasmid 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 either by:-

a) linearisation of the plasmid with a single cutting enzyme which does not cut within the target insert. For the plasmids used by the UK NRL, linearise using *Eco*RI enzyme (HAV) or *Xba*I enzyme (norovirus GI and GII).

b) PCR amplification of an amplicon spanning the target region using a dilute solution of the plasmid as template. For the plasmids used by the UK NRL, the use of the pTAG 5 and pTAG 3 primers is recommended (see Appendix 1 for sequences).

Following generation of linear control DNA, a small amount should be cleaned up using e.g. a commercial PCR purification kit. The concentration of DNA can then 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 $5x10^{-8}$ (and by any dilution factor involved) will give the concentration of DNA in g/µl.

Divide this number by the mass in g of a single dsDNA molecule to calculate the concentration of DNA in copies/µl (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 (6.02×10^{23}) e.g. a molecule of 3000bp will have a mass of 3.02×10^{-18}g).

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

HAV	$3.41x10^{-18}$g	(3383 bp)
Norovirus GI	$3.32x10^{-18}$g	(3287 bp)
Norovirus GII	$3.32x10^{-18}$g	(3292 bp)

For the pTAG PCR products amplified from the plasmids used by the UK NRL the masses are as follows:-

HAV	$3.41x10^{-19}$g	(338 bp)
Norovirus GI	$2.45x10^{-19}$g	(242 bp)
Norovirus GII	$2.50x10^{-19}$g	(247 bp)

The preparation of dsDNA should then be diluted with a suitable buffer (e.g. TE buffer) to a concentration of approximately $1x10^4$ -$1x10^5$ copies/µl, and stored in single use aliquots at 4°C for up to 24 h, 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.

Double-stranded DNA control plasmids as described above are used for the production of EC RNA. These 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, a small amount should be linearised using a suitable restriction enzyme (to enable linearization of the plasmid at a point shortly downstream of the target sequence) and buffers as recommended by the manufacturer of the enzyme. For the plasmids used by the UK NRL, linearise using *Eco*RI enzyme (HAV EC RNA) or *Xba*I enzyme (norovirus GI and GII EC RNA). The reaction should then be cleaned up using e.g. a commercial PCR purification kit.

EC RNA should be transcribed from 1 µg 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 used by the NRL, EC RNA can be in vitro transcribed using the SP6/T7 Riboprobe combination system (**Promega**, see Riboprobe® Combination Systems 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 1µg/µl)	5 µl
T7 polymerase (for norovirus GI/GII EC RNA)	3 µl

OR

SP6 polymerase (for HAV EC RNA)	3 µl
Nuclease free water	39.5 µl

Mix by pipetting

2. Incubate for 2 hours at 37ºC.
3. Add 5 µl RQ1 RNase-free DNase to the reaction.
4. Incubate for 15 min at 37ºC.

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 https://www.qiagen.com/gb/ for information, cat nos. 74104, 74106] using the manufacturer's RNA cleanup protocol) and eluting in 100µl 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® mastermix where RT has been deactivated by heating at 95ºC, and untreated mastermix. 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.

Multiplication of the A260 value by $4x10^{-8}$ (and by any dilution factor involved) will give the concentration of RNA in g/µl.

Divide this number by the mass in g of a single EC RNA molecule molecule to calculate the concentration of DNA in copies/µl (the mass of an individual RNA molecule may 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 (6.02×10^{23}) e.g. an RNA molecule of 200 ribonucleotides will have a mass of 1.06×10^{-19} g

For the EC RNAs used by the UK NRL the masses are as follows:-

Norovirus GI	$6.73x10^{-20}$g	(126 b)
Norovirus GII	$7.00x10^{-20}$g	(131 b)
HAV	$1.33x10^{-19}$g	(250 b)

The preparation of RNA transcripts should then be diluted with a suitable buffer (e.g. TE buffer) to a concentration of approximately $1x10^4$ -$1x10^5$ transcripts/µl, and stored in single use aliquots at 4°C for up to 24 h, 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 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 µl per sample plus 25 µl excess).

Retain a 20 µl subsample of pooled material for RNA extraction and preparation of the standard curve. Store at 4 ºC 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.

Select a minimum of 10 live, or if frozen, undamaged animals (sufficient shellfish to produce 2g 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 chop finely with a razor blade.

Transfer a 2g 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 2g 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 µl of mengo virus process control material directly onto the 2g 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 60ºC 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 consisting of 500 µl water should be extracted in parallel.

For each test sample, add 2 ml of NucliSens lysis buffer to a tube. For each sample add 500 µl of the supernatant produced in 7.1 to one tube containing lysis buffer 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 (from the Nuclisens magnetic extraction reagents) and resuspend the pellet by pipetting/vortexing.

7.2.1 RNA extraction using the miniMAG equipment

Transfer suspension to a 1.5 ml screw-cap tube. Wash for 30 s using the automated wash steps of the miniMAG. After washing allow silica to settle using magnet of the miniMAG. Discard supernatant by e.g. aspiration.

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

Separate tubes from magnet, then add 500 µl wash buffer 2 (from the Nuclisens magnetic extraction reagents). Resuspend pellet, wash for 30 s, allow silica to settle using magnet then discard supernatant. Repeat.

Separate tubes from magnet, then add 500 µl wash buffer 3 (from the Nuclisens magnetic extraction reagents). Wash for 15 s, 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 (from the Nuclisens magnetic extraction reagents). Cap tubes and transfer to thermoshaker or equivalent.

Incubate for 5 min at 60 ºC with shaking at 1400 rpm.

Place tubes in magnetic rack and allow silica to settle, then transfer eluate to a clean tube and retain at 4 ºC for a maximum of 24 h or -20 ºC for longer periods (up to 6 months).

7.2.2 RNA extraction without using the miniMAG equipment

Transfer suspension to a clean 1.5 ml tube. Cap tubes and wash for 30 s by vortexing. After washing, allow silica to sediment using the magnetic rack. Discard supernatant by e.g. aspiration.

Add 400 µl wash buffer 1. Cap tubes and wash silica for 30 sec by vortexing, allow silica to sediment using magnetic rack then discard supernatant.

Add 500 µl wash buffer 2 (from the Nuclisens magnetic extraction reagents). Cap tubes and wash silica for 30 s by vortexing, allow silica to sediment using magnetic rack then discard supernatant. Repeat.

Add 500 µl wash buffer 3 (from the Nuclisens magnetic extraction reagents). Cap tubes and wash silica for 15 s by vortexing, allow silica to sediment using magnetic rack then discard supernatant.

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

Add 100 µl elution buffer (from the Nuclisens magnetic extraction reagents). Cap tubes and transfer to thermoshaker or equivalent.

Incubate for 5 min at 60 ºC with shaking at 1400 rpm.

Place tubes in magnetic rack and allow silica to settle, then transfer eluate to a clean tube and retain at 4ºC for a maximum of 24 h or -20ºC for longer periods (up to 6 months).

7.3 TaqMan® analysis – general requirements

Note: results generated using 10^{-1} 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^{-1} 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® 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® mastermixes immediately before starting procedure.

7.4 TaqMan® 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® mastermix to each well.

7.5 TaqMan® plate set-up - analysis of mengo virus

For each batch of mengo virus process control material extracted (7.3) prepare 10^{-1}, 10^{-2} and 10^{-3} dilutions of mengo virus RNA in water or 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® mastermix to each well.

See layout on following page for example TaqMan® plate testing one sample for all three targets.

Example plate layout (single sample – all assays on one plate) NOTE: This layout includes wells with 10^{-1} 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	H2O + HAV EC RNA	-ve extraction control	-ve extraction control	H_2O	H2O	
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	H2O + GI EC RNA	-ve extraction control	-ve extraction control	H_2O	H2O	
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	H2O + GII EC RNA	-ve extraction control	-ve extraction control	H_2O	H2O	
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)	-ve extraction control	H_2O				

HAV assay
Norovirus GI assay
Norovirus GII assay
Mengo virus assay

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

7.6 TaqMan® assay run parameters

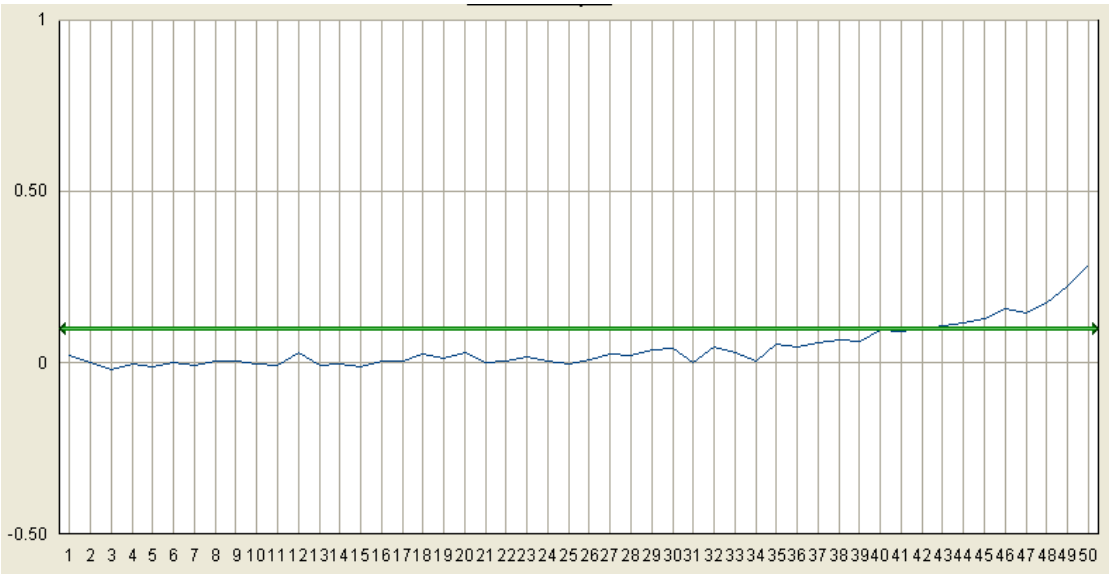
Run the TaqMan® assay with the following parameters:-

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	45
	Annealing-extension	60 °C for 1 min 65 °C for 1 min	

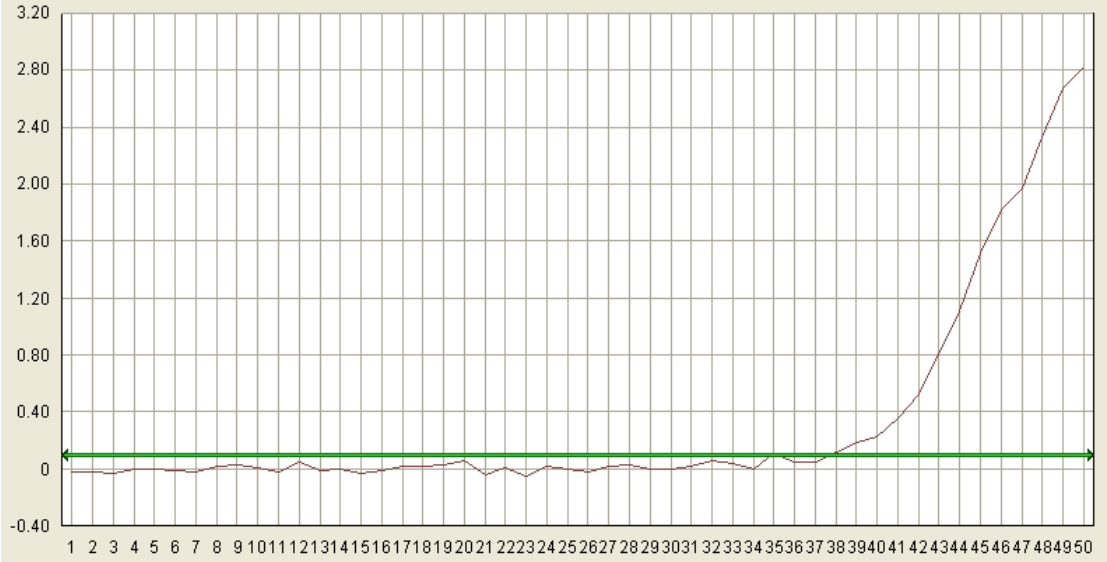
7.7 Analysis of results

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).

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.



e.g. in this case the recorded C_q value was 34.92, however it should be noted by the analyst that the correct figure should be e.g. 38.

Use the C_q values of each dilution series to create standard curves for each control by plotting the C_q values obtained against $\log_{10}$ concentration (e.g. $\log_{10}$ copies per microlitre target dsDNA) to determine r^2, slope and intercept parameters. Do not average C_q values from duplicate reactions prior to plotting.

Curves with r^2 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 C_q values of the standard curve for any outlying values and remove these from the series. No more than two such outlying C_q values should be removed per series and values from a minimum of three (mengo virus RNA) or four dilutions (dsDNA) must be retained.

Repeat the calculations to determine r^2, slope and intercept parameters. Where the modified curve has an r^2 value of <0,980, or where the slope is not between -3,10 and -3,60, the modified slope should not be used for calculations.

Note: subject to the existence of supporting data, laboratories can apply more stringent acceptance criteria for r^2 (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:-

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

where ΔC_q = C_q value [sample RNA + EC RNA] - C_q value [water + EC RNA] and where m = slope of the dsDNA standard curve.

A sample (+ EC RNA) producing the same Cq values as the water + EC RNA reaction 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^{-1} sample RNA + EC RNA wells for the same target. If the RT-PCR inhibition level using the 10^{-1} RNA is <75%, results for the 10^{-1} RNA should be used

for that sample and target. If RT-PCR inhibition levels for both undiluted and 10^{-1} 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^{-1} 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^{-1} sample RNA results are used multiply by 10 to correct for the dilution factor):-

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

where ΔC_q = C_q value [sample RNA] – C_q value [undiluted process control virus RNA] and where m = slope of the mengo 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^{-1} dependent on the RT-PCR inhibition results; see above) and use these to calculate target concentrations (in detectable virus genome copies/µl RNA) ***for each replicate*** by reference to the relevant dsDNA standard curve as follows:-

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

where ΔC_q = C_q value [sample RNA] – standard curve intercept and where m = slope of the dsDNA standard curve.

Negative replicates should be given a concentration of zero copies/µl RNA. For each sample calculate the average of the concentrations for both replicates.

Multiply this value by 200 (undiluted RNA) or 2 000 (10^{-1} 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.

Positive results for each target virus should be expressed as **"x detectable virus genome copies per g"** where x is the calculated concentration, provided that this level is above the limit of quantification (LOQ) of the method (the LOQ is the lowest concentration of target in a test sample that can be quantified with an acceptable level of precision and accuracy).

If target RNA is detected at levels <LOQ, these should be expressed as **"virus genome detected at levels below the limit of quantification"** followed by **"(y detectable virus genome copies per gram)"** where y is the LOQ of the method.

If target virus is not detected results should be expressed as **"not detected"** followed by **"(<z detectable virus genome copies per gram)"** where z is the limit of detection (LOD) of the method (the LOD is the lowest concentration of target in a test sample that can be reproducibly detected with 95% confidence).

If a valid result is not obtained, results should normally be expressed as **"invalid"**. If however, an otherwise valid positive result is obtained from a sample showing an unacceptable RT-PCR inhibition or extraction efficiency, results may, if appropriate, be expressed as **"virus genome detected in 2 g"**.

Sampling is not considered in this protocol. It should be noted that absence of virus in the sample under test may not guarantee absence of virus in an entire consignment.

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.

da Silva AK, Le Saux JC, Parnaudeau S, Pommepuy M, Elimelech M, Le Guyader FS. 2007. Evaluation of removal of noroviruses during wastewater treatment, using Real-Time Reverse Transcription-PCR: different behaviors of genogroups I and II. Appl Environ Microbiol. 73(24):7891-7897.

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.

Kageyama T, Kojima S, Shinohara M, Uchida K, Fukushi S, Hoshino FB, Takeda N, Katayama K. 2003. Broadly reactive and highly sensitive assay for Norwalk-like viruses based on real-time quantitative reverse transcription-PCR. J Clin Microbiol. 41(4):1548-1557.

Le Guyader FS, Parnaudeau S, Schaeffer J, Bosch A, Loisy F, Pommepuy M, Atmar RL. 2009. Detection and quantification of noroviruses in shellfish. Appl Environ Microbiol. 75(3):618-624.

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.

Svraka S, Duizer E, Vennema H, de Bruin E, van der Veer B, Dorresteijn B, Koopmans M. 2007. Etiological role of viruses in outbreaks of acute gastroenteritis in The Netherlands from 1994 through 2005. J Clin Microbiol. 45(5):1389-1394.

10.0 Appendix 1: 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]
TM9 (PROBE):	TGG ACA GGA GAT CGC	[Hoehne & Schreier, 2006]

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

HAV

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

pTAG (for pGEM series plasmids)

pTAG 5:	GCT ATG ACC ATG ATT ACG CCA A	[Maguire et al., 1999]
pTAG 3:	TGT AAA ACG ACG GCC AGT GAA	[Maguire et al., 1999]

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