Generic Protocol

UK National Reference Laboratory for foodborne viruses

Detection of norovirus and hepatitis A virus on surfaces

Issue 1 - March 2019

GENERIC PROTOCOL

Detection of norovirus and hepatitis A virus on surfaces

17 pages

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Issue No.	**1**
Date	**08/03/19**

1.0 Introduction

Norovirus (NoV) and hepatitis A virus (HAV) are important agents of food-borne human viral illness. While some food-borne transmission relates to contamination of the foodstuff at primary production, other outbreaks occur due to contamination of the surfaces of foods or food preparation surfaces by for example infected food handlers. No routine methods exist to culture these viruses from food matrices and surfaces. 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. For surfaces, viruses are removed by swabbing. RNA is then 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 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 qualitative detection of viral RNA from the test sample.

2.0 Scope

This procedure describes collection, purification and detection of NoV genogroups I (GI) and II (GII) and HAV, from surfaces (the surfaces of foods, or food preparation surfaces). 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 qualitative detection of viral RNA from the test sample.

3.0 Principle

3.1 Virus collection

Viruses are collected from surfaces by swabbing. 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 assay has consequently a high sensitivity and specificity.

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, 1000µl, 200µl, 20µl and 10µl.
- Pipette filler.
- Pipettes of a range of sizes, 25 ml, 10 ml, 5 ml.
- Vortex mixer.
- Thermoshaker operating at 60 ºC and 1400 rpm or equivalent.
- Aspirator or equivalent apparatus for removing supernatant.
- Bench centrifuge and rotor capable of running at 1,500 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 and 50 ml.
- Sterile cotton swabs.
- 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

- Sodium chloride (NaCl)
- Potassium chloride (KCl)
- Disodium hydrogen phosphate (Na_2HPO_4)
- Potassium dihydrogen phosphate (KH_2PO_4)
- Tris base
- Ethylenediaminetetraacetic acid (EDTA) disodium dihydrate
- NucliSens magnetic extraction reagents. **BioMerieux**. See http://www.biomerieux.com/ for information. Cat numbers; 200293 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.

- **Phosphate buffered saline (PBS)**

  Add 8 g NaCl, 0.2 g KCl, 1.15 g Na_2HPO_4, 0.2 g KH_2PO_4 and 1000 ml molecular grade water to a bottle. Mix with stirring until the solids are dissolved. Sterilise by autoclaving. Adjust the pH to 7.3. Store at 2-6 °C for up to 6 months. Alternatively use PBS from a commercial source.

- **Tris solution (1 M)**

  Add 12.1 g Tris base and 80 ml molecular grade water to a bottle. Mix with stirring until the solids are dissolved. Adjust the volume to 100 ml with molecular biology grade water. 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 80 ml molecular biology grade water to a bottle. Adjust the volume to 100 ml with molecular biology grade water 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 1000 µl 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 20µl/reaction and mix by vortexing.

- **Hepatitis A virus Taqman® PCR buffer**

  Add the following reagents to a 1.5ml 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 20 µl/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 10000) 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 3,000 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.

- **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.

  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; www.promega.com/tbs/tb086/tb086.pdf) at a *Hinc*II 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 OCLs 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.

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 http://www.promega.com/catalog/country_select.asp?/default.asp&ckt=2 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 mins 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. 74103, 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 95 ºC, 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 frozen in single use aliquots.

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

7.0 Method

7.1 Virus collection

Premoisten a sterile cotton swab with PBS, then use this to intensively swab the test surface (maximum area, 100 cm^2), applying a little pressure to detach virus particles. Where practical, record the approximate area swabbed in square centimetres.

Process the swab immediately, or place in a suitable container and store at 4 °C for a maximum of 72 h, at −15 °C or below for up to 6 months, or at −70 °C or below for longer periods.

Immediately before any batch of swabs is processed, pool together sufficient aliquots of Mengo virus process control material for use with all swabs (allow 10 µl per swab 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.

Add 10 µl of Mengo virus process control virus material to the swab, then immediately immerse the swab in a tube containing 490 µl Nuclisens lysis buffer. Press the swab against the side of the tube to release liquid. Repeat the immersion and pressing cycle three or four times to ensure maximum yield of virus.

Proceed immediately to RNA extraction (7.2).

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. Add the entire sample 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 (e.g. by 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 400 µl 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 s 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 4 ºC for a maximum of 24 h or -20 ºC 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 unacceptable 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 unacceptable 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 (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.

Add 20 µl of the relevant TaqMan® mastermix to each well

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 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.

7.5 TaqMan® 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 water or a suitable buffer (e.g. TE buffer).

Add 20 µl of the Mengo virus TaqMan® mastermix to each well.

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 5µl 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.

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)

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	H_2O	
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	H_2O	
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	H_2O	
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

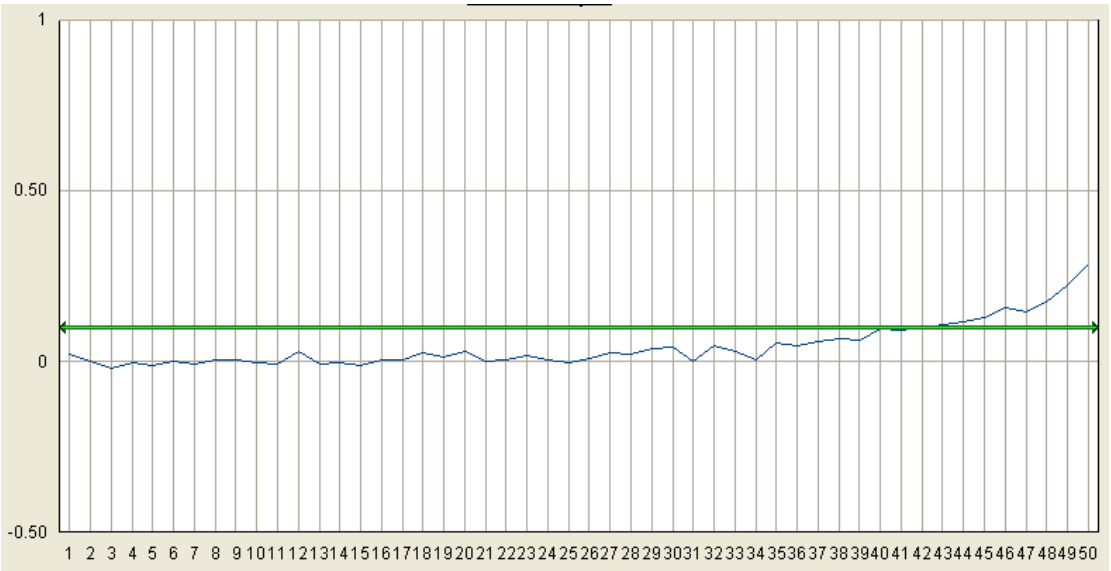
Run the TaqMan® assay with the following parameters:-

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	

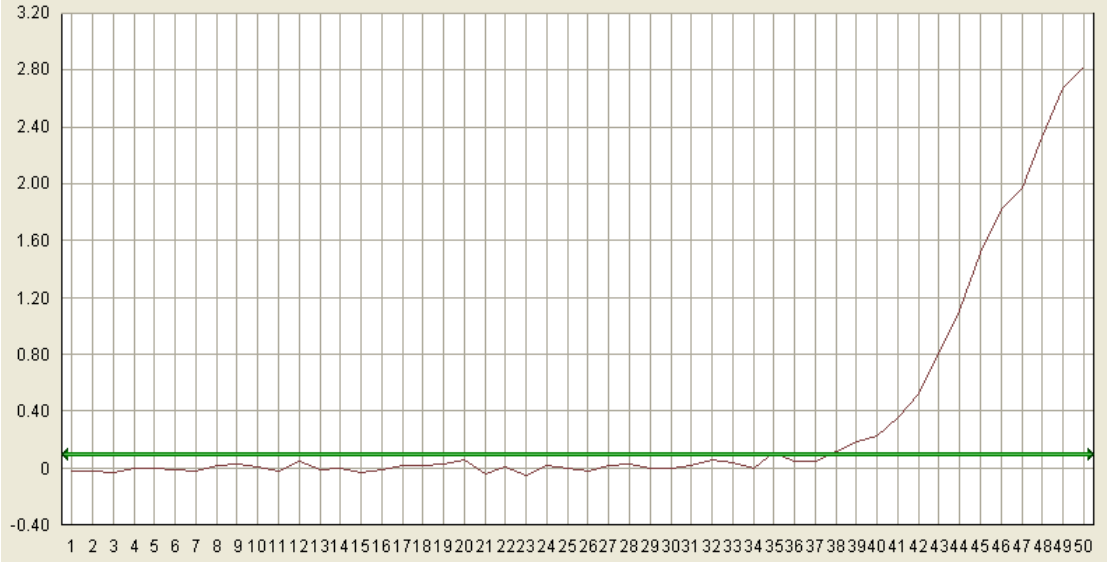
7.7 Analysis of results

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 Cq 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 Mengo virus RNA dilution series to create standard curves 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.

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 one such outlying C_q values should be removed per series and values from a minimum of three dilutions 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

Check for RT-PCR inhibition by comparing the Cq values for the sample RNA + EC RNA wells with the Cq value for the water + EC RNA well as follows. If the Cq value of the undiluted sample RNA + EC RNA well is < 2.00 greater than the Cq value of the water + EC RNA well, results for the undiluted RNA should be used for that sample. If the Cq value of the undiluted sample RNA + EC RNA well is ≥ 2.00 greater than the Cq value of the water + EC RNA well, repeat the comparison with the 10^{-1} sample RNA + EC RNA well.

If the Cq value of the 10^{-1} sample RNA + EC RNA well is < 2.00 greater than the Cq value of the water + EC RNA well, results for the 10^{-1} RNA should be used for that sample. If the Cq value of the 10^{-1} sample RNA + EC RNA well is ≥ 2.00 greater than the Cq value of the water + EC RNA well, sample 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 extraction efficiency 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):-

Extraction efficiency = $10^{(\Delta Cq/m)}$ x 100%

where ΔC_q = C_q value [sample RNA] – C_q value [undiluted process control virus RNA]

and where m = slope of the dsDNA standard curve

A sample producing the same Cq value as undiluted Mengo virus RNA will have an extraction efficiency of 100%. Where the extraction efficiency is <1% sample results are not valid and the sample should be retested.

For each sample with acceptable RT-PCR inhibition level and extraction efficiency, results for each target can be determined by looking at results for the appropriate sample RNA only wells.

Where a Cq is determined in either duplicate well the test result for the sample is positive and should be expressed as **"virus genome detected in x cm^2"** where the approximate surface area x could be recorded or as **"virus genome detected"** where the approximate surface area x could not be recorded. Where no Cq is determined in either duplicate well the test result for the sample is not detected and should be expressed as **"virus genome not detected in x cm^2"** or **"virus genome not detected"** as appropriate.

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 not detected in x cm^2"** or **"virus genome not detected"** as appropriate.

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' 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

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