Protocol - Qualitative detection of norovirus and hepatitis A virus in soft fruit. Issue 2: 23.11.12

Cefas
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GENERIC PROTOCOL

Qualitative detection of norovirus and hepatitis A virus in soft fruit

Issued by Technical Manager, Shellfish Virology

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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 soft fruit such as raspberries and strawberries. 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. For soft fruit virus extraction is by elution with agitation followed by precipitation with PEG/NaCl. 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 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 document enables qualitative detection of virus RNA in the test sample.

Note: this procedure was developed by a group of expert European laboratories contributing to the standardisation of methods for detection of viruses in foodstuffs (CEN TC275/WG6/TAG4). The laboratory protocol given here is compliant with the pending Technical Specification CEN/ISO TS 15216-2 due for publication in 2013. Prepublication drafts of this technical specification may be available from national standardisation bodies. Other laboratory protocols compliant with the Technical Specification CEN/ISO TS 15216 may also be considered as acceptable for detection of norovirus and hepatitis A virus in soft fruit. It should be noted that Cefas scientists have limited direct experience with the soft fruit matrix processing aspects of this protocol.

2.0 Scope

This procedure describes liberation, concentration and quantification of NoV genogroups I (GI) and II (GII) and HAV, from soft fruit (including raspberries and strawberries). 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 part of the procedure describes a method for qualitative detection of virus RNA in the test sample.

3.0 Principle

3.1 Virus extraction

Viruses are eluted from soft fruit under alkaline conditions. Viruses are then concentrated by PEG/NaCl precipitation and finally the virus extract is cleaned using chloroform:butanol. 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. An increase of fluorescence above a threshold level is indicative of the presence of target RNA in the test 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, 1000μl, 200μl, 20μl and 10μl.
- Pipette filler.
- Pipettes of a range of sizes, 25ml, 10ml, 5ml.
- Vortex mixer.
- Thermoshaker operating at 60°C and 1400 rpm or equivalent.
- Aspirator or equivalent apparatus for removing supernatant.
- Heating block capable of operating at 95°C or equivalent.
- Mesh filter bags (400 ml).
- pH meter (or pH testing strips).
- Rocking platform(s) or equivalent for use at room temperature and 4°C at 60rpm.
- Bench centrifuge and rotor capable of running at 1,500 x g with capacity for 15/50ml tubes.
- Refrigerated centrifuge(s) and rotor(s) capable of running at 10000 x g with capacity for tubes for ≥ 20 ml and for narrow gauge (15 mm is too large) chloroform resistant tubes.
- Microcentrifuge.
- Centrifuge and microcentrifuge tubes/bottles of a range of sizes, 1.5ml, 15ml, 50ml etc. narrow gauge (15 mm is too large) chloroform resistant tubes with 1 ml capacity are necessary.
- 1.5ml tubes with screw caps suitable for use with the miniMAG/easyMAG extraction systems.
- 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
- Polyethylene Glycol (PEG), MW 8000
- Sodium chloride (NaCl)
- Potassium chloride (KCl)
- Disodium hydrogen phosphate (Na₂HPO₄)
- Potassium dihydrogen phosphate (KH₂PO₄)
- Tris base
- Glycine
- Beef extract powder
- Pectinase from Aspergillus niger or Aspergillus aculeatus
- Chloroform
- Butanol
- Sodium hydroxide (NaOH)
- Hydrochloric acid (HCl)
- NucliSens magnetic extraction reagents. BioMerieux. See http://www.biomerieux.com/ for information. Cat numbers; 200293 etc.
- Nuclease free water

6.2 Prepared solutions/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 mastermix. For other manufacturers consult the machine instructions.

- 5x PEG/NaCl solution (50% (w/v) PEG 8000, 1.5M NaCl)
  Add 500g PEG 8000, 87g NaCl and 450ml molecular grade water to a bottle. Mix with gentle shaking/stirring, heating gently if necessary, until the solids are dissolved then adjust the volume to 1000ml. Sterilise by autoclaving.

- Chloroform:Butanol
  Add together equal volumes of chloroform and butanol. Shake to mix.

- Phosphate buffered saline (PBS)
  Add 8g NaCl, 0.2g KCl, 1.15g Na₂HPO₄, 0.2g KH₂PO₄ and 1000ml molecular grade water to a bottle. Mix with stirring until the solids are dissolved. Sterilise by autoclaving. Adjust the pH to 7.3. Alternatively use PBS from a commercial source.
• **Tris glycine 1% beef extract (TGBE) buffer**

  Add 12.1g Tris base, 3.8g glycine, 10g beef extract powder and 1000ml molecular grade water to a bottle. Mix with stirring until the solids are dissolved. Adjust the pH to 9.5. Sterilise by autoclaving.

• **Norovirus GI 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 ROX Reference Dye (1 x or 0.1 x; 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 20μl/reaction and mix by vortexing.

• **Norovirus GII 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 ROX Reference Dye (1 x or 0.1 x; see above) (from Ultrasense system)
  - 12.5 pmol/reaction QNIF2 (FWD) primer
  - 22.5 pmol/reaction COG2R (REV) primer
  - 6.25 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

  - 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 ROX Reference Dye (1 x or 0.1 x; 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 1.5ml 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 ROX Reference Dye (1 x or 0.1 x; 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 20μl/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 CO₂ levels, cell culture consumables (flasks etc.) and media.

  Mengo virus strain MC0 (ATCC VR-1597) should be used unless proscribed by e.g. GMO regulations. In this case wild-type mengo virus (ATCC VR-1598) can be used. Mengo virus should best be grown in a 5% CO₂ 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
  
  2mM L-glutamine
  Earle's BSS, adjusted to
  
  1.5g/l sodium bicarbonate
  0.1mM non-essential amino acids
  1.0mM 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
  
  4mM L-glutamine, adjusted to
  
  1.5g/l sodium bicarbonate
  4.5g/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 -80°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.

- **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 EU-RL 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) at a *Hincl* restriction site such that the target sequence was downstream of a promoter sequence for the SP6 RNA polymerase. 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. The EU-RL may be able to supply these plasmids to NRLs upon request. [The EU-RL is also looking at the development of commercially available dsDNA controls].
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 EU-RL, 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 100-500 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 used by the EU-RL, 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: 20μl
   - 100 mM DTT: 10μl
   - RNasin: 2.5μl
   - rATP,rGTP,rCTP,rUTP mix (2.5mM each): 20μl
   - linearised template DNA (max 1μg/μl): 5μl
   - T7 polymerase (for norovirus GI/GII EC RNA): 3μl
   - OR
   - SP6 polymerase (for HAV EC RNA): 3μl
   - Nuclease free water: 39.5μl
   Mix by pipetting
   2. Incubate for 2 hours at 37°C.
   3. Add 5μl RQ1 RNase-free DNase to the reaction.
   4. Incubate for 15 mins at 37°C.

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/SelectCountry.aspx 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°C, 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 EU-RL 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 nuclease-free water to a concentration of approximately 1x10^{6} -1x10^{8} transcripts/μl, and frozen in single use aliquots.

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 10μl per sample plus 25μl excess).

Dilute a 20μl subsample of pooled material to 10^{-1} using nuclease-free water and store (for use in TaqMan®) at 4°C for a maximum of 24 hrs or in single-use aliquots at -20°C for longer periods.

Coarsely chop 25g of soft fruits into pieces of approximately 2.5 cm × 2.5 cm × 2.5 cm (it is not necessary to chop if e.g. individual fruits are smaller than this) and transfer to the sample compartment of a 400 ml mesh filter bag.

Add 40ml TGBE buffer with pectinase (30 units of pectinase from Aspergillus niger, or 1140 units of pectinase from Aspergillus aculeatus) and 10μl of mengo virus process control virus material.

Incubate at room temperature with constant rocking at approximately 60rpm for 20min. For acidic soft fruits, the pH of the eluate should be monitored at 10 min intervals during incubation. If the pH falls below 9.0 it should be adjusted to 9.5 with NaOH. Extend the period of incubation by 10 min for every time the pH is adjusted. Decant the eluate from the filtered compartment into a centrifuge tube (use two tubes if necessary to accommodate volume).

Clarify by centrifugation at 10000 x g for 30 min at 4°C.

Decant the supernatant into a single clean tube/bottle and adjust the pH to 7.0 with HCl.

Add 0.25 volumes of 5 × PEG/NaCl solution (to produce a final concentration of 10 % PEG 0.3M NaCl), homogenise by shaking for 60s then incubate with constant rocking at around 60 rpm at 5°C for 60min.

Centrifuge at 10000 x g for 30min at 5°C (split volume across two centrifuge tubes if necessary).

Decant and discard the supernatant, then centrifuge at 10000 x g for 5min at 5°C to compact pellet.

Discard the supernatant and resuspend pellet in 500μl PBS. If a single sample has been split across two tubes resuspend both pellets stepwise in the same aliquot of PBS.

Transfer suspension to a narrow gauge chloroform resistant centrifuge tube. Add 500μl chloroform-butanol, vortex to mix, then incubate at room temperature for 5 min.
Centrifuge at 10000 x g for 15 min at 5°C. Carefully transfer the aqueous phase to a fresh tube and retain for RNA extraction.

7.2 RNA extraction

**Note:** for every set of samples a negative extraction control consisting of 500μl water should be extracted in parallel.

Add 2ml of NucliSens lysis buffer to a tube. Add the 500μl sample produced in 7.1 and mix by vortexing briefly.

Incubate for 10 min at room temperature.

Add 50μl 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 400μl wash buffer 1 and resuspend the pellet by pipetting/vortexing.

Transfer suspension to a 1.5ml 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 400μl wash buffer 1. Resuspend pellet, wash for 30 sec, allow silica to settle using magnet then discard supernatant.

Separate tubes from magnet, then add 500μl 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 500μl 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 100μl 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 at 4°C for a maximum of 24 hrs or -20°C for longer periods (up to 6 months).

7.3 TaqMan® analysis – general requirements

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.

For each sample and each target assay add 5μl of undiluted and 10⁻¹ sample RNA to two wells of the plate each.

For each negative extraction control and each target assay add 5μl of undiluted RNA to one well.
For each target assay add 5μl of nuclease-free water to two wells.
For each target assay add 1μl of 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 20μl of the relevant TaqMan® mastermix to each well.

7.5 TaqMan® plate set-up - analysis of mengo virus
Defrost one aliquot of diluted (10⁻¹) mengo virus process control material for each batch used with the samples under test.
Heat at 95°C for 5min using a heating block or equivalent to release RNA. using a heating block or equivalent.
Chill tubes rapidly, centrifuge at ≥ 3000 x g for 1min., then transfer the supernatant ("mengo virus RNA") to a fresh tube.
Prepare 10⁻¹, 10⁻² and 10⁻³ dilutions of mengo virus RNA in nuclease free water for each batch of mengo virus process control.
Add 5μl of undiluted and 10⁻¹ sample RNA to one well of the plate each.
For each negative extraction control add 5μl 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 5μl of nuclease-free water to one well.
Add 20μl 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)

<table>
<thead>
<tr>
<th>Test sample (undiluted)</th>
<th>Test sample (-1)</th>
<th>Test sample (undiluted) + GI EC RNA</th>
<th>Test sample (-1) + GI EC RNA</th>
<th>H2O + GI EC RNA</th>
<th>-ve extraction (–ve process control)</th>
<th>H2O</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test sample (undiluted)</td>
<td>Test sample (-1)</td>
<td>Test sample (undiluted) + GII EC RNA</td>
<td>Test sample (-1) + GII EC RNA</td>
<td>H2O + GII EC RNA</td>
<td>-ve extraction (–ve process control)</td>
<td>H2O</td>
</tr>
<tr>
<td>Test sample (undiluted)</td>
<td>Test sample (-1)</td>
<td>Test sample (undiluted) + HAV EC RNA</td>
<td>Test sample (-1) + HAV EC RNA</td>
<td>H2O + HAV EC RNA</td>
<td>-ve extraction (–ve process control)</td>
<td>H2O</td>
</tr>
<tr>
<td>Test sample (undiluted)</td>
<td>Test sample (-1)</td>
<td>Process control virus RNA (undiluted)</td>
<td>Process control virus RNA (-1)</td>
<td>Process control virus RNA (-2)</td>
<td>Process control virus RNA (-3)</td>
<td>-ve extraction (–ve process control)</td>
</tr>
</tbody>
</table>

Norovirus GI assay
Norovirus GII assay
HAV assay
Mengo virus assay

5μl RNA (+/- 1μl EC RNA) & 20μl mastermix per well
7.6 TaqMan® assay run parameters

Run the TaqMan® assay with the following parameters:

<table>
<thead>
<tr>
<th>Step description</th>
<th>Temperature and time</th>
<th>Number of cycles</th>
</tr>
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<tbody>
<tr>
<td>RT</td>
<td>55 °C for 1 h</td>
<td>1</td>
</tr>
<tr>
<td>Preheating</td>
<td>95 °C for 5 min</td>
<td>1</td>
</tr>
<tr>
<td>Amplification</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Denaturation</td>
<td>95 °C for 15 s</td>
<td></td>
</tr>
<tr>
<td>Annealing-</td>
<td>60 °C for 1 min</td>
<td>45</td>
</tr>
<tr>
<td>extension</td>
<td>65 °C for 1 min</td>
<td></td>
</tr>
</tbody>
</table>

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

Check Cq values of the process control virus RNA standard curve dilution series for any points that do not fall close to the line of best fit. These Cq values should not be incorporated into standard curve calculations.

Use the remaining Cq values of each dilution series to create a standard curve. Points from a minimum of 3 dilutions must be included. Curves with r² values of <0.98 should not be used for calculations.

To determine the amplification efficiency for each sample and each target refer to the Cq values for the wells containing EC RNA. 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, results may not be valid and the sample may need to be retested.

Use the Cq value for the mengo virus assay from the test sample RNA well (undiluted or 10⁻¹ dependent on the amplification efficiency results; see above) to estimate extraction efficiency by reference to the mengo virus RNA standard curve (if 10⁻¹ sample RNA results are used multiply by 10 to correct for the dilution factor). 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 may need to be retested.

For each sample with acceptable amplification and extraction efficiency, results for each target can be determined by looking at results for the appropriate sample RNA only well. Where a Cq is determined the test result for the sample is positive and should be expressed as “virus genome detected in x g” where x is the amount of sample tested. Where no Cq is determined the test result for the sample is not detected and should be expressed as “virus genome not detected in x g” where x is the amount of sample tested.

If a valid result is not obtained results should normally be expressed as “no result”. If however, an otherwise valid positive result is obtained from a sample showing an unacceptable amplification or extraction efficiency, results may be expressed as detailed above. Details should be included in the test report.

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


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]

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

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

NOTE: the probe sequence above is that recommended by CEN TC275/WG6/TAG4. Cefas has had better results in bivalve shellfish samples with the following norovirus GI probe:-

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

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

Either probe sequence is compliant with the pending Technical Specification ISO/CEN TS 15216-2.

Norovirus GII

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

COG2R (REV): TCG ACG CCA TCT TCA TCA 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 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