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Standard Operating Procedure

for

Qualitative detection of norovirus and hepatitis A virus on surfaces

Version No.	Date	Comments
1	2022-04-27	Based on ISO 15216-2:2019 Microbiology of the food chain — Horizontal method for determination of hepatitis A virus and norovirus using real-time RT-PCR Part 2: Method for detection

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1.0 Introduction

Norovirus and hepatitis A virus (HAV) are important agents of food-borne human viral illness. Surfaces (food surfaces, food preparation surfaces and food contact surfaces) are associated with transmission of norovirus and HAV. No routine methods exist to culture these viruses from surfaces. Detection is therefore reliant on molecular methods using the reverse-transcriptase polymerase chain reaction (RT-PCR). As surfaces may 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. Virus extraction from surfaces is done by intensively swabbing with a cotton swab premoistened in PBS. Viral RNA is extracted by immersing the cotton swab in lysis buffer containing chaotropic reagents followed by adsorption of RNA to silica particles.

Real-time RT-PCR monitors amplification throughout the PCR cycle by measuring the excitation of fluorescently labelled molecules. In the 5'-fluorogenic nuclease-based real-time RT-PCR assay the fluorescent labels are attached to a sequence-specific nucleotide probe (hydrolysis probe) that also enables simultaneous confirmation of target template. These modifications increase the sensitivity and specificity of the PCR method, and obviate the need for additional amplification product confirmation steps post PCR. Due to the complexity of the method, it is necessary to include a comprehensive suite of controls. The method described in this document enables qualitative detection of virus RNA in the test sample.

Note: The laboratory protocol given here is based on ISO 15216-2:2019 with some modifications.

2.0 Scope

This procedure describes liberation, concentration and detection of norovirus genogroups I (GI) and II (GII) and HAV, from surfaces. Surface is defined as surface of food, food preparation surface or food contact surface. 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 lifted from the surface by using a sterile cotton swab premoistened in PBS by intense swabbing of the surface. The cotton swab is transferred to lysis buffer for subsequent RNA extraction.

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 steps. 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. 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 is measured at each cycle throughout the run. 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.

Both NoV and HAV are class 2 pathogens, highly infectious and as little as 1-100 virus particles can cause disease. Be sure to wash hands with soap and water and sterilize work surfaces with DAX 70+ or daily fresh 10 % chlorine solution. Ethanol is not effective against NoV or HAV.

Mengo virus is a murine virus that belongs to the Picornaviridae family. The mengo virus string MC0 (ATCC VR-1957) is a mutant, where large parts of the wild type Poly(C) region is deleted. This causes the phenotype to become avirulent, but retains all growth properties.

5.0 Recommended equipment

- Sterile cotton swabs
- Micropipettes.
- Micropipette tips of a range of sizes, 1000 µl, 200 µl, 100 µl and 10 µl.
- Centrifuge tubes/bottles of a range of sizes, 1.5 ml, 15 ml.
- Vortex mixer.
- Thermoshaker operating at 60 ºC and 1400 rpm or equivalent.
- Aspirator or equivalent apparatus for removing supernatant.
- Centrifuge and rotor capable of the following run speed, and rotor capacity:
    - 1 500 × g with capacity for 15 ml centrifuge tubes.
- RNA extraction equipment suitable for extraction using guanidine thiocyanate disruption and silica adsorption-based method.
- If miniMAG/easyMAG extraction system is used; 1.5 ml tubes with screw caps suitable for this system.
- PCR machine with real-time capacity capable of supporting TaqMan® chemistry.
- Consumables for real-time PCR, e.g. optical plates and caps.

6.0 Reagents

6.1 Reagents used as purchased

- Phosphate-buffered saline (PBS)
- Sodium chloride (NaCl)
- Potassium chloride (KCl)
- Disodium hydrogen phosphate (Na_2HPO_4)
- Potassium dihydrogen phosphate (KH_2PO_4)
- If NucliSens system is used; magnetic extraction reagents. See Biomerieux website http://www.biomerieux.com/
- If NucliSens system is used; lysis buffer. See Biomerieux website http://www.biomerieux.com/ for information.
- RNA UltraSense™ One-Step Quantitative RT-PCR System. Applied Biosystems™.
- 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 ºC 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).

- **Phosphate buffered saline (PBS)**

  Add 8 g NaCl, 0.2 g KCl, 1.15 g Na_2HPO_4, 0.2g 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 ± 0.2. Alternatively use PBS 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)
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)
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

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)
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)
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 CO_2 levels, cell culture consumables (flasks etc.) and media.

  Mengo virus[1] 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

  0.1mM 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

[1] Mengo virus strain MC_0 is recommended. It is a genetically modified organism (GMO). MC_0 has the same growth properties as wild type but has an avirulent phenotype. In laboratories where use of GMO is prohibited or problematic, a different process control virus should be used.

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

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

  A detailed standard operating procedure for the production of EC RNA can be found in document "Eurl standard operating procedure for preparation of dsdna and ecrna.pdf"at Livsmedelsverkets website https://www.livsmedelsverket.se

  Control plasmids used in the development of ISO/TS 15216-2 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 *Hin*cII 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 *Sma*I restriction site such that in each case the target sequence was downstream of a promoter sequence for the T7 RNA polymerase.

  The EURL constructed control plasmids that can be provided by the EURL. All plasmids contain synthesised target sequences with an upstream T7 promoter sequence and a downstream *Hin*dIII restriction site cloned into a pEX-A2 vector (Eurofins Genomics, Ebersberg, Germany).

  - Inserted target sequence for HAV control plasmid corresponds to nucleotide 54 to 299 of the HAV genome (GenBank accession number, NC001489), with an inserted *Bam*HI restriction site.
  - Inserted target sequence for norovirus GI control plasmid corresponds to nucleotide 5215-5452 of the norovirus GI genome (GenBank accession number, M87661.2), with an inserted *Bam*HI restriction site.
  - Inserted target sequence for norovirus GII control plasmid corresponds to nucleotide 4935-5180 of the Lorsdale virus genome (GenBank accession number, X86557.1), with an inserted *Bam*HI restriction site.

  Alternatively, separate control plasmids for each target virus can be constructed by individual laboratories 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 control plasmids constructed at the EURL and the production of EC-RNA from these plasmids are described in detail in document "Eurl standard operating procedure for preparation of dsdna and ecrna.pdf" at Livsmedelsverkets website https://www.livsmedelsverket.se
  For the plasmids used in the development of ISO/TS 15216-2, linearize 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 linearized plasmid DNA using an in-vitro RNA transcription reaction mix prepared as recommended by the manufacturer of the relevant RNA polymerase enzyme. Following incubation,

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

For the plasmids described here, EC RNA can be in vitro transcribed using the SP6/T7 Riboprobe combination system (Promega website http://www.promega.com/. For information, cat no. P1460) as follows:

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

5X transcription buffer	20 µl
100 mM DTT	10 µl
RNasin	2.5 µl
rATP,rGTP,rCTP,rUTP mix (2.5mM each)	20 µl
linearised template DNA (max 1µg/µl)	5 µl
T7 polymerase	3 µl
OR	
SP6 polymerase	3 µl
Nuclease free water	39.5 µl

Mix by pipetting

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

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 Qiagen website http://www1.qiagen.com/, 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 or a fluorometer.

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

Divide this number by the mass in g of a single EC RNA molecule to calculate the concentration of DNA in copies/µl (the mass of an individual RNA molecule can be calculated by multiplying the RNA length in ribonucleotides by 320.5 (the molecular weight of an average ribonucleotide) and dividing by the Avogadro constant (6.02×10^{23}) e.g. an RNA molecule of 200 ribonucleotides will have a mass of 1.06×10^{-19} g.

Examples for masses of two of the RNA produced from plasmids described above:-

Norovirus GI ISO	$6.71x10^{-20}g$	(126 b)
Norovirus GII EURL	$1.29x10^{-19}g$	(243 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

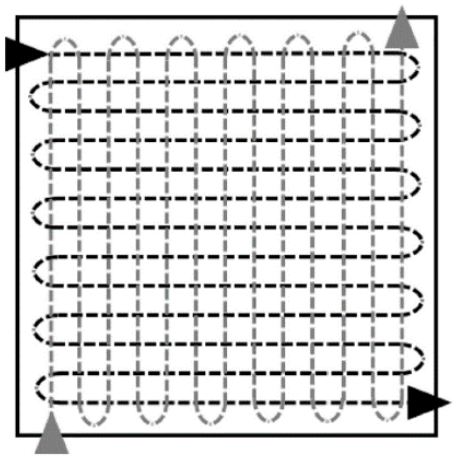
7.1 Virus extraction

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 hrs or at -20 ºC for longer periods.

Moisten a cotton swab in PBS.

Swab intensively with light pressure, according to the figure. Swabbed area should not exceed 100 cm^2. Record approximate area swabbed in cm^2.



Process the swab directly, or place it in a suitable container for storage at 4 °C for 72 h, at -20 °C for a maximum of 6 months, or at -70 °C for longer periods.

Pipette 10 µl of process control virus (mengo virus) to the swab.

Immediately after the addition of process control virus, immerse the swab in a tube with 490 µl Lysis Buffer as used for RNA extraction, then press against the side of the tube to release liquid. Repeat the immersion and pressing cycle three times.

Prepare a Negative Process Control (NPC) by moistening a sterile cotton swab in PBS and dip it directly in 500 µl Lysis Buffer with subsequent rinsing according to

the paragraph above. Do not swab any surface and do not add any process control virus to the NPC swab.

7.2 RNA extraction

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

Note: Below is the NucliSens protocol from bioMerieux described. If an equivalent method is used follow the manufacturar's instructions.

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

Incubate for 10 min at room temperature.

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

Incubate for 10 min at room temperature.

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

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

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

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

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

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

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

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

Incubate for 5 min at 60 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 hrs or -20ºC for longer periods (up to 6 months).

7.3 Inhibitor removal kit (not included in ISO 15216)

For samples suspected to be unclean and inhibitory, the use of an inhibitor removal kit can diminish inhibition. Inhibitory substances from food surfaces

could be substantial and the use of inhibitory removal kits should be based on previous experiences.

The EURL has experience of OneStep™ PCR Inhibitor Removal Kit (Zymo Research).

Follow the manufacturer's instructions.

7.4 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 process control virus 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.

7.5 TaqMan® plate set-up - analysis of target viruses

Note: this section describes plate set-up for a single target virus according to ISO 15216-2. At the EURL we add an extra well for sample and 10^{-1} dilutions. The EURL also use 2 µl of EC RNA instead of 1 µl stated in ISO 15216-2.

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 5 µl of undiluted and 10^{-1} sample RNA to two wells of the plate each.

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

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

For each target assay add 1 µl 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 20 µl of the relevant TaqMan® mastermix to each well.

7.6 TaqMan® plate set-up - analysis of process control virus

Note: this section describes plate set-up for mengo virus process control material according to ISO 15216-2. At the EURL we add an extra well for sample, 10^{-1} dilution, negative extraction control and nuclease free water but not for the mengo virus dilution series.

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

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

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

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

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

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

See layout on following page for example TaqMan® plate testing one sample for all three targets. The plate-set-up follows the ISO 5126 set up, but the EURL recommends addition of wells as described in notes for 7.5 and 7.6. Especially is this true for samples with suspected low level of target virus.

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) + GI EC RNA	Test sample (-1) + GI EC RNA	H2O + GI EC RNA	NPC or NEC	NPC or NEC	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	NPC or NEC	NPC or NEC	H_2O	H_2O	
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	NPC or NEC	NPC or NEC	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)	NPC or NEC	H_2O				

Norovirus GI assay
Norovirus GII assay
HAV assay
Mengo virus assay

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

7.7 TaqMan® assay run parameters

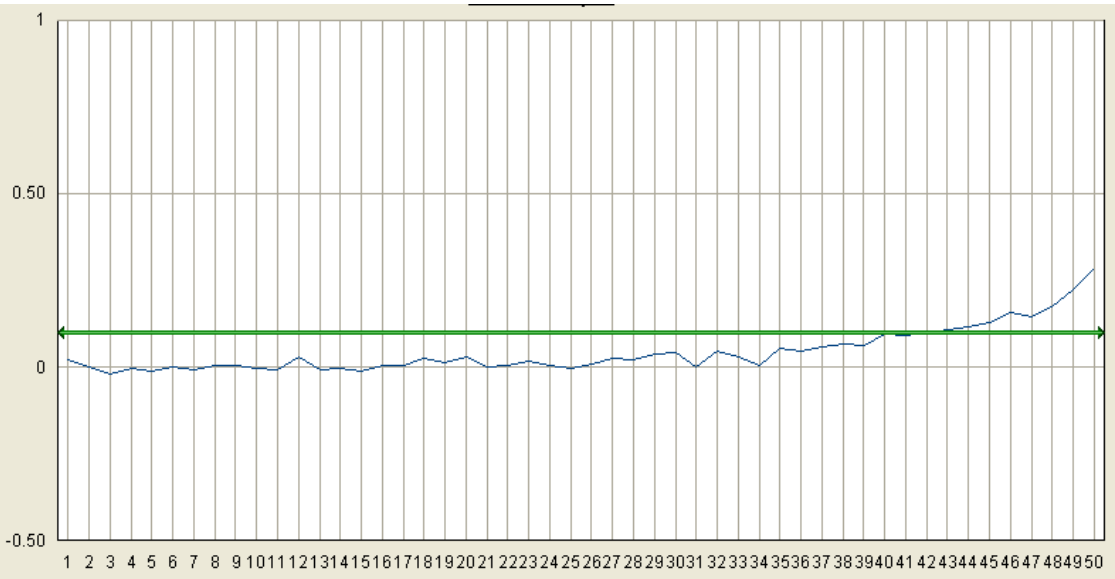
Run the TaqMan® assay with the following parameters:

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	

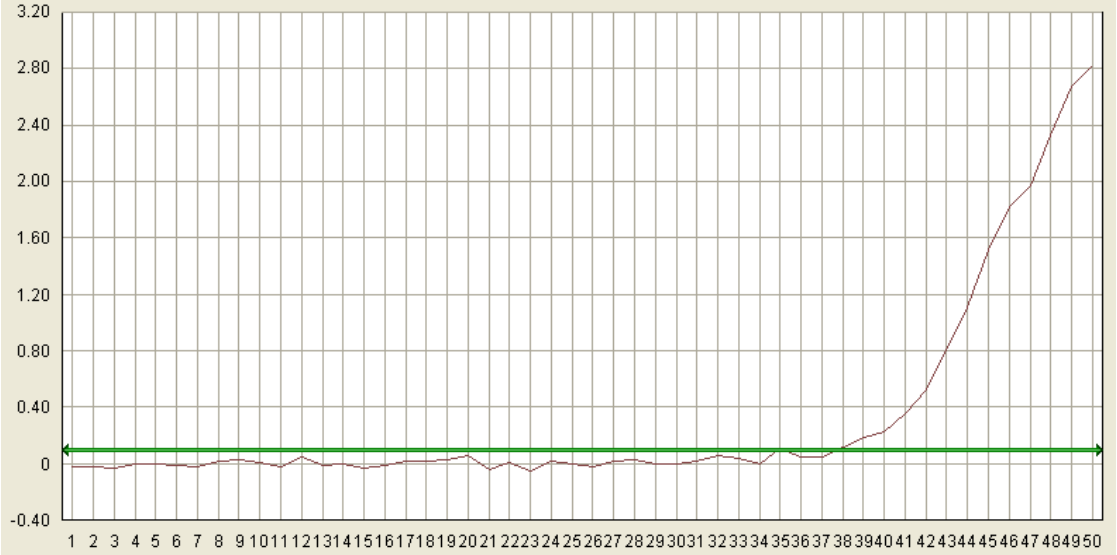
7.8 Analysis of results

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

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



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



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

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. For each dilution series points from a minimum of 3 dilutions must be retained.

Use the remaining Cq values of each dilution series to create a standard curve by plotting the Cq values obtained against $\log_{10}$ concentration 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.

To determine the RT-PCR inhibition 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 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):-

Process control virus recovery = $10^{(\Delta Cq/slope)}$ x 100%

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

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 RT-PCR inhibition level 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 cm^2"**. 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 cm^2"**.

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 RT-PCR inhibition level 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

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10.0 Appendix 1: Primer and probe sequences described in ISO 15216

Norovirus GI

QNIF4 (FW):	CGC TGG ATG CGN TTC CAT	[da Silva et al., 2007]
NV1LCR (REV):	CCT TAG ACG CCA TCA TCA TTT AC	[Svraka et al., 2007]

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

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

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

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

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

Norovirus GII

QNIF2 (FW):	ATG TTC AGR TGG ATG AGR TTC TCW GA	[Loisy et al., 2005]
COG2R (REV):	TCG ACG CCA TCT TCA TTC ACA	[Kageyama et al., 2003]
QNIFS (PROBE):	AGC ACG TGG GAG GGC GAT CG	[Loisy et al., 2005]

Probe labelled 5' FAM, 3' TAMRA

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