Bacteriological Analytical Manual
Chapter 26
Concentration, Extraction and Detection of Enteric Viruses from Food

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Revision History: Chapter 26B has been archived and all sections have been updated and extended to include the RT-qPCR analysis of multiple food matrices for hepatitis A virus and norovirus GI and GII. We would like to acknowledge Gary Hartman for his work with Chapter 26B. Chapter 26A has also been archived.

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Introduction

Virus associated outbreaks due to the consumption of contaminated food occur annually in the US and nearly all of these outbreaks are due to human norovirus and/or hepatitis A virus (HAV) (1). Detection of viruses in implicated foods is challenging because of the low level of viral contamination, inefficient extraction from food matrices, and the inability to enrich viruses-an aspect beneficial to most bacterial methodologies. These factors necessitate that isolated virus particles be sufficiently concentrated in order to detect their presence in foods. Such limitations can hinder surveillance and outbreak investigations. Inherently, advances in molecular detection techniques such as reverse transcription (RT)-PCR and real-time RT-q(quantitative)PCR (2, 3) have been shown to offer specificity and sensitivity for foodborne pathogen detection.

The development of virus concentration methods utilizing ultracentrifugation combined with innovative extraction and molecular based detection methods has been successful in virus research, foodborne virus outbreaks, and surveillance studies. Ultracentrifugation, in combination with varying buffers containing amino acids to dissociate viruses from the food is the premise for the FDA foodborne virus concentration and extraction methodologies described within this chapter. These produce and seafood methods, apart from the molluscan shellfish method, utilize a rinse step prior to concentration. The principle of these methods allows the recovery of intact, potentially infectious virus particles which can be assessed for viability. In addition, specific steps are included in each method to reduce the presence of substances which can cause inhibition of molecular based detection methods. Efficient methods for detection of enteric viruses in these food matrices are important for outbreak response and as such, these methods focus on produce and seafood, the leading commodities associated with foodborne enteric virus outbreaks (4).

The concentration and extraction protocols are coupled with RT-qPCR detection using established primers and hydrolysis probes (5, 6, 7, 8). The norovirus, HAV, and murine norovirus (extraction control) RT-qPCR assays were developed and validated for use on the Cepheid SmartCycler® and AB 7500 platforms. The Smart Cycler platform is no longer in use and the protocols are available in Appendix I and the archived Chapter 26B. The norovirus RTqPCR assay simultaneously detects human norovirus genogroups I and II (GI and GII). In addition, a mengovirus multiplex RT-qPCR assay was developed for use as an alternate extraction control (to murine norovirus) and is included for analysis on the AB 7500. All assays incorporate an internal amplification control to prevent the reporting of false negatives results due to inhibition or failure of the RT-qPCR. These multiplexed detection assays were validated for the qualitative detection of norovirus GI, GII, and HAV nucleic acids and can be used for detection of these viruses from any food matrix. Valid sample results are contingent upon the detection of the murine norovirus or mengovirus extraction control from the sample being tested for norovirus GI, GII, and/or HAV. The validation results, data analysis, and supplemental

material associated with the concentration, extraction, and detection assays are available in the appendices.

I. Materials and Reagents for the Concentration, Extraction, and Detection of Enteric Viruses

1. 95% Ethanol (Sigma E7023 or equivalent)
2. DNase/RNase free water (Life Technologies AM9937 or equivalent)
3. Deionized/Distilled water
4. NaCl (Sigma S3014 or equivalent)
5. NaOH (Sigma S5881 or equivalent)
6. HCl (Sigma H1758 or equivalent)
7. Glycine (Sigma G7126 or equivalent)
8. Threonine (Sigma T8375 or equivalent)
9. Guanidine Isothiocyante (Fisher Scientific 15535-016 or equivalent)
10. Chloroform(Sigma C2432 or equivalent)
11. 6 N HCl (Sigma XX0628 or equivalent)
12. 3 N HCl (Fisher Scientific 18-610-903 or equivalent)
13. pH strips (Fisher Scientific 88-841 or equivalent)
14. 10X PBS tissue culture (t.c.) grade (Sigma P5493) to be diluted to 1X
15. KCl (Sigma P9541 or equivalent)
16. Potassium Dihydrogenphosphate (Sigma P9791 or equivalent)
17. Disodium Hydrogen Phosphate (Sigma S5011 or equivalent)
18. Glycerol (Sigma G5516 or equivalent) ***Reagent specific for Section A5***
19. Potassium Citrate (Sigma P1190 or equivalent) ***Reagent specific for Section A2***
20. Beef extract powder (Sigma B4888 or equivalent) ***Reagent specific for Section A2***
21. Tris Base (Fisher Scientific BP152-1 or equivalent) ***Reagent specific for Section A2***
22. Pectinase (Fisher Scientific ICN19897910 from *Aspergillus niger* or equivalent) ***Reagent specific for Section A2***
23. DNase/RNase-free microcentrifuge tubes, non-stick, low retention, siliconized 0.5 ml (Life Technologies AM12350 or equivalent)
24. DNase/RNase-free microcentrifuge tubes 1.5 ml, non-stick, low retention, siliconized (Life Technologies AM12450 or equivalent)
25. DNase/RNase-free microcentrifuge tubes 2.0 ml, non-stick, low retention, siliconized (Life Technologies AM12475 or equivalent)
26. Filter barrier aerosol resistant micropipettor tips DNase/RNase free (0.2 – 1000 µl)
27. Qiagen QIAamp Viral RNA Mini Kit (Qiagen 52904)
28. Qiagen RNeasy Mini Kit (Qiagen 74104) ***Reagent specific for Section A3***

29. Qiagen QIAshredder (Qiagen 79654) ***Reagent specific for Section A1, A2, A4***
30. Qiagen collection tubes (Qiagen 19201)
31. OneStep™ PCR Inhibitor Removal Kit (Zymo Research D6030)
32. 2.0 mL microcentrifuge tubes DNase/RNase free (USA Scientific 1620-2799 or equivalent)
33. Primer TE (formula provided Section A6)
34. OneStep RT-PCR Kit (Qiagen 210210 or 210212)
35. Ambion Superase·In RNase Inhibitor (20 units/µl); Life Technologies AM2694 (2,500 U) or AM2696 (10,000 U)
36. 50 mM $MgCl_2$ (BioRad 1708872 or equivalent) or 25 mM $MgCl_2$ (ThermoFisher Scientific AB0359, or equivalent)
37. Internal Control RNA (BioGX Cat No. 750-0001—contact company)
38. Positive controls (Quantitative Synthetic Norovirus GI— ATCC VR-3234SD, Quantitative Synthetic Norovirus GII—ATCC VR-3235SD, HAV—ATCC VR-1402 and murine norovirus ATCC VR 1937)
39. Fluorescein calibration dye (BioRad 1708780 or equivalent)
40. Extraction control murine norovirus (ATCC VR 1937)
41. Mengovirus (CECT Spanish Type Culture Collection vMCO CECT 100000 Spanish ATCC) (https://www.uv.es/uvweb/spanish-type-culture-collection/en/news/cect-incorporates-mengovirus-vmco-public-catalogue-1285923246228/Novetat.html?id=1286062095701)
42. Standard desalted primers and HPLC probes for all RTqPCR assays (Integrated DNA Technologies or equivalent)

II. Equipment and Supplies for the Concentration, Extraction, and Detection of Enteric Viruses

1. pH meter (Cole Parmer EW 53026-54 or equivalent)
2. Biological Safety Cabinet (BSC- 2 Type A2 or higher air exchange rate)
3. Ultra low freezer (-70 ˚C or lower)
4. Latex or nitrile gloves (powder-free)
5. Vortex mixer (Labsource S16-109 or equivalent)
6. Nasco Whirl Pak™ filter bags;6” x 9” (Fisher Scientific B01348WA or equivalent)
7. DNase/RNase-free microcentrifuge tubes, non-stick, low retention, siliconized 0.5 ml (Life Technologies AM12350 or equivalent)
8. Sterile scissors
9. Ice Bucket and crushed ice
10. Hype-Wipe Disinfecting Towelettes (Fisher Scientific 14-412-56 or equivalent)
11. Adjustable Micropipettors (0.2 – 1000 µl), dedicated for RNA work only

12. Filter barrier aerosol resistant micropipettor tips DNase/RNase free (0.2 – 1000 µl)
13. AB 7500 FAST (Applied BioSystems 4351106)
14. ABI 96 well plates (Life Technologies 4346906)
15. ABI plates cover (Life Technologies 4311971)
16. Mini Plate Spinner (Fisher Scientific 14-100-143 or equivalent)
17. Mini Centrifuge (Labsource C90-044 or equivalent)
18. 96 well cool rack (Sigma Aldrich #Z606634-1EA or equivalent)
19. 500 ml Blender (Cole Parmer EW 04243-25 or equivalent) ***Equipment specific for Section A3***
20. Blender Base (Fisher 14-509-19, or equivalent) ***Equipment specific for Section A3***
21. Disposable scalpels (Fisher Scientific 12-460-456 or equivalent) ***Supplies specific for Section A3***
22. 50 ml polypropylene conical tubes (Fisher Scientific 14-959-49A, or equivalent)
23. 150 mm x 15 mm Petri Dishes (Fisher 08-757-148 or equivalent)
24. 50 mL serological pipettes (Labsource K79-156-CS or equivalent)
25. Mettler Toledo™ NewClassic ME Precision Balance, 2200 g (Fisher Scientific 01-912-408 or equivalent)
26. Drummond pipette aid (Fisher Scientific or equivalent)
27. Refrigerated centrifuge capable of speeds of up to 12,000 x g, and with rotors capable of holding 50 ml conical tubes (ThermoFisher Scientific 75006591or equivalent)
28. FY14 50 carbon fiber rotor (ThermoFisher Scientific 46922 or equivalent)
29. 70 ml polypropylene ultracentrifuge tubes (Fisher Scientific 01-013-33 or equivalent)
30. Ultracentrifuge capable of speeds of 170,000 x g and with rotors capable of holding 70 ml ultracentrifuge tubes (ThermoFisher Scientific 75000100 or equivalent)
31. Centrifuge capable of speeds of 21,000 x g, and with rotors capable of holding 1.5 ml and 2.0 ml microcentrifuge tubes (Eppendorf 2231000655 or equivalent)
32. Disposable transfer pipettes (Fisher 13-711-22 or equivalent)
33. Orbital shaker (Fisher Scientific 11-676-231 or equivalent)

SECTION A: VIRUS CONCENTRATION AND EXTRACTION

Section A1: Concentration and Extraction of Enteric Viruses from Green Onion and Leafy Greens

1. Tare and weigh 50 g ± 2 g of produce cut in 2-5" pieces into a Whirl-Pak filter bag.

Note: Include all parts of the produce present including roots to leaves.

2. Add 100 µl extraction control to sample (Section A5).
3. Add 55 mL ± 2 ml of Glycine Buffer pH 7.6 (Section A6) and seal.
4. Shake at 200 rpm for 15 min at room temperature.
5. Pipette liquid into a clean 50 ml conical tube. Let bag sit for 2-3 min and pipette remaining liquid into the same 50 ml conical tube.

Note: Do not squeeze bag to obtain more buffer. This will lead to RT-qPCR assay inhibition.

6. Centrifuge at 9,000 x *g* for 30 min at 4 ± 2 ºC.
7. Pipette supernatant into a clean 70 ml Ultra-centrifuge tube being careful not to disturb pellet and other debris.
8. Bring total volume up to 65 ml or 125 g total weight (includes bottle and cap) with addition Glycine Buffer, pH 7.6.
9. Balance tubes to within 0.05 g of each other using Glycine Buffer, pH 7.6.

Note: Minimum volume for ultracentrifugation using Fiberlite rotor and tubes is 50 ml, add glycine/NaCl buffer to bring volume to ≥ 50 ml.

10. Centrifuge 170,000 x *g* for 60 min at 4 ± 2 ºC.
11. Slowly decant supernatant (should see pellet on side of tube), let tubes sit for 4-5 min, then remove and discard remaining liquid with a disposable transfer pipette.
12. Add 280 µl of Glycine Buffer pH 7.6 to tube and use a disposable transfer pipette to carefully resuspend sample (do not draw all contents at once into pipette). Evenly distribute sample into two 2.0 ml DNase/RNase free tubes.

Note: If sample pellet plus buffer is larger than 0.5 ml, refer to Qiagen QIAamp Viral RNA Mini Kit manual instructions on how to proceed with larger samples volumes.

13. Store these sample concentrates at ≤ -70 °C or proceed directly to RNA extraction.

Note: Only 1 tube is required for RNA extraction. The remaining tube should be stored at ≤ -70 °C as reserve for possible future analysis.

RNA Extraction

Note: Before starting RNA extraction warm buffer AVE in a 70 ºC heating block.

1. Add 560 µl prepared Buffer AVL with carrier RNA (Section A6) to sample concentrate tube (if concentrate is frozen, allow thawing at room temperature).
2. Incubate at room temperature (15-25 °C) for 10 min.
3. Resuspend pellet by pipetting up and down and vortexing.
4. Transfer 700 µl of supernatant to the QIAshredder column.
5. Centrifuge 2 min at maximum speed (≥ 16,000 x g) in a microcentrifuge.
6. Carefully transfer the supernatant of the flow-through fraction to a new 2.0 ml low retention/siliconized DNase/RNase free microcentrifuge tube without disturbing the cell-debris pellet (if present) in the collection tube.
7. Add 560 µl of 95-100% ethanol to the cleared lysate, close tube and mix immediately by inverting 3 times. Do not centrifuge. Continue without delay to next step.
8. Apply 630 µl of the solution to an QIAamp mini column.
9. Centrifuge 8,000 x *g* for 1 min. Place QIAamp spin column in new collection tube. Discard flow through and collection tube.
10. Continue to add sample until the entire sample has been passed through the column, discarding the collection tube each time.
11. Transfer the QIAamp mini column into a new 2 ml collection tube. Add 500 µl Buffer AW1. Centrifuge 1 min at 8,000 x *g*. Discard flow through and collection tube.

12. Transfer the QIAamp mini column into a new 2 ml collection tube. Pipet 500 µl Buffer AW2 onto the QIAamp Mini column. Centrifuge at maximum speed (≥ 16,000 x g) for 3 min. Discard flow through and collection tube.

13. Transfer the QIAamp mini column into a new 2 ml collection tube. Centrifuge at maximum speed (≥ 16,000 x *g*) for 1 min to dry column.

14. To elute RNA, transfer the QIAamp mini column in to a new 1.5 ml low retention/siliconized DNase/RNase free centrifuge tube. Add 50 µl of pre-heated (70 ºC) Buffer AVE directly onto the QIAamp silca-gel membrane. Close the tube gently, and centrifuge for 1 min at 8,000 x *g*.

15. Add an additional 50 µl of pre-heated Buffer AVE to column. Pipette the eluted 50 µl back to the top of the column. Close the tube gently, and centrifuge for 1 min at 8,000 x *g*.

16. Proceed with RT-qPCR or freeze at ≤ -70 °C for storage.

Section A2: Concentration and Extraction of Enteric Viruses from Soft Fruit: Fresh and Frozen

1. Tare and weigh 50 g ± 5 g of soft fruit (fresh or frozen) to a Whirl-Pak filter bag using sterile forceps or scoop.

Note: Remove pectinase from refrigerator and allow to come to room temperature

2. Add 100 µl extraction control to sample (extraction control preparation Section A5).

3. Add 30 ml of Glycine Beef Extract (Section A6).

4. Add 50 µl of room temperature pectinase (Section A6).

5. Tightly close bag and invert 3 times to cover fruit with buffer.

Note: Do not squeeze bag to obtain more buffer. This will cause RT-qPCR inhibition.

6. Shake at 150 rpm for 15 min at room temperature.

7. Check pH - sample pH should be ≥ 7.5, if pH is below 7.5, adjust to 8.0 ± 0.5 using NaOH (2.5 M or 5 M).

8. Pipette liquid into a clean 50 ml conical tube. Let bag sit for 2-3 min and pipette remaining liquid into the same 50 ml conical tube.

9. Centrifuge at 12,000 x g for 15 min at 4 ± 2 ºC.

10. Pipette supernatant into a clean 70 ml Ultra-centrifuge tube being careful not to disturb pellet and other debris.

11. Bring total volume up to 65 ml or 125 g total weight (includes bottle and cap) with addition of t.c. PBS (Section A6).

12. Balance tubes to within 0.05 g of each other using t.c. PBS.

13. Centrifuge at 170,000 x g for 45 min at 4 ± 2 ºC.

Note: Minimum volume for ultracentrifugation using Fiberlite rotor and tubes is 50 ml.

14. Slowly decant supernatant, discard excess liquid with disposable transfer pipette.

Note: If there is gelatinous substance present, gently tap side of ultra-tube with hand to dislodge the gelatinous substance and remove with micro-pipettor.

15. Add 600 µl of t.c. PBS to the Ultra-centrifuge tube. Using a disposable transfer pipette, carefully resuspend sample (do not draw all contents at once into pipette).

16. Transfer resuspended concentrate into one 2.0 ml DNase/RNase free tube.

17. Vortex for 60 ± 5 sec.

18. Add 800 µl of chloroform to tube.

19. Vortex for 60 ± 5 sec.

20. Centrifuge at 3,000 x g for 5 min at 4 ± 2 ºC.

21. Using a disposable transfer pipette, evenly distribute aqueous layer into three separate 2.0 ml DNase/RNase free microcentrifuge tubes.

22. Store these concentrates at ≤ -70 °C or proceed directly to RNA extraction.

Note: Only 1 tube is required for RNA extraction. The remaining tubes should be stored at -70 °C as reserve for future analysis.

RNA Extraction

Note: Before starting extraction, prepare AVL with carrier RNA and place and warm an aliquot of Buffer AVE in 70 ºC heating block.

1. Add 560 µl prepared Buffer AVL with carrier RNA (Section A6) to sample concentrate tube (if concentrate is frozen, allow thawing at room temperature).
2. Vortex for 20 sec and incubate at room temperature (15-25 °C) for 10 min.
3. Add 100 µl 2 M Potassium Acetate solution (Section A6) and invert 3 times.
4. Incubate on ice for 15 min.
5. Centrifuge for 10 min at 4 ± 2 ºC at maximum speed (≥ 16,000 x *g*).
6. Carefully remove all of supernatant and transfer to 2 ml DNase/RNase free centrifuge tube (do not disturb pellet, if present).
7. Transfer 700 µl of supernatant to a QIAshredder column.
8. Centrifuge at maximum speed (≥ 16,000 x g) for 2 min in a microcentrifuge.
9. Carefully transfer the supernatant of the flow-through fraction to a new 2.0 ml low retention/siliconized DNase/RNase free microcentrifuge tube without disturbing the cell-debris pellet (if present) in the collection tube.
10. Repeat steps 7-9 using additional QIAshredder columns, if necessary, until entire sample has been processed through a QIAshredder. Transfer the flow through to the 2 ml microcentrifuge tube in step 9.
11. Add 700 µl of 95-100% ethanol to the flow through and mix immediately by inverting. Do not centrifuge. Continue without delay to next step.
12. Apply 630 µl of the solution to a QIAamp mini column.
13. Centrifuge 6000 x *g* for 1 min. Place the QIAamp spin column in a new collection tube. Discard flow through and collection tube.
14. Repeat steps 12 and 13 until the entire sample has been passed through the column, discarding the collection tube each time.
15. Transfer the QIAamp mini column into a new 2 ml collection tube. Add 500 µl Buffer AW1. Centrifuge 1 min at 6000 x *g*. Discard flow through and collection tube.
16. Transfer the QIAamp mini column into a new 2 ml collection tube.
17. Add 500 µl Buffer AW2 onto the QIAamp mini column. Centrifuge at maximum speed (≥ 16,000 x *g*) for 3 min. Discard flow through and collection tube.

18. Transfer the QIAamp mini column into a new 2 ml collection tube. Centrifuge at maximum speed (≥ 16,000 x *g*) for 1 min to dry column.

19. To elute RNA, transfer the QIAamp mini column into a new 1.5 ml low retention/siliconized DNase/RNase free centrifuge tube.

20. Add 50 µl of pre-heated (70 ºC) Buffer AVE directly onto the QIAamp silca-gel membrane. Close the tube gently, and centrifuge for 1 min at 8000 x g.

21. Pipette the eluted 50 µl back to the top of the column. Add an additional 50 µl of pre-heated Buffer AVE to column. Close the tube gently, and centrifuge for 1 min at 8,000 x *g*.

22. Discard column and place tube with RNA (step 21) on ice to prepare Zymo columns.

23. Prepare two Zymo columns per manufacture's instructions.

24. Transfer Zymo column into a clean 1.5 or 2.0 ml low-retention/siliconized RNase/DNase free microcentrifuge tube.

25. Transfer RNA from step 21 to prepared Zymo One Step RT-PCR inhibitor remover column.

26. Spin at 8,000 x *g* for 3 min.

27. Repeat by adding recovered RNA to a freshly prepared Zymo spin column.

28. Proceed with RT-qPCR or freeze at -70 °C for storage.

Section A3: Concentration and Extraction of Enteric Viruses from Molluscan Shellfish

Note: In order to preserve RNA viruses, the shellfish sample should be kept on ice whenever possible during the process. To avoid any RNA degradation, DNase/RNase-free microcentrifuge tubes should be used.

1. Place 12 shucked whole oysters (minus the adductor muscle) in a petri dish and dissect out the digestive gland Appendix C).

Note: If samples are frozen, allow thawing at room temperature prior to dissecting.

2. In a separate petri dish, mince the digestive glands with scalpel and weigh a 4 ± 0.5 g in a sterile 50 ml conical tube.

3. Add 100 µl extraction control.

4. Add 40 ml sterile deionized water (volume added should equal to 10 times weight of oyster meat).

5. Gently mix and transfer entire contents to blender and blend for 45 ± 15 sec, ensuring that the sample is completely homogenized.

6. Return homogenate into the same 50 ml conical tube.

7. Adjust the pH of the homogenate to 4.5 ± 0.5 with the addition of HCl (3 N or 6 N). Check pH using a pH meter or strips (add 2 to 3 drops of homogenate to the strip or if using hand held pH meter add drops to a clean petri dish and check pH - do not add contents back to sample).

8. Centrifuge the homogenate at 2,000 x *g* for 15 min at 4 ± 2 ºC.

9. Decant and discard supernatant (viruses are should be adsorbed to the shellfish pellet at this point).

10. Resuspend pellet with 40 ml of Glycine buffer pH 7.6 (Section A6) and shake vigorously to bring into solution.

11. Adjust the pH of the homogenate to 7.5 ± 0.3 with the addition of NaOH (2.5 M or 5 M). Check pH using a pH meter or strips (add 2 to 3 drops of homogenate to the strip or if using hand held pH meter add drops to a clean petri dish and check pH - do not add contents back to sample).

12. Centrifuge the homogenate at 5,000 x *g* for 15 min at 4 ± 2 ºC.

13. Transfer supernatant/eluate into a 70 ml ultracentrifuge tube and place tube on ice.

14. Resuspend pellet in 20 ml of Threonine buffer and shake vigorously to bring into solution.

15. Centrifuge at 5,000 x *g* for 15 min at 4 ± 2 ºC.

16. Collect supernatant/eluate and combine into same 70 ml ultracentrifuge tube from step 13.

17. Weigh and balance ultracentrifuge tubes with caps and contents to within 0.05 g of each other using t.c. PBS.

18. After balancing, centrifuge for 1 hr at 170,000 x *g*.

19. Discard supernatant and resuspend pellet in 5 ml of t.c. PBS. Transfer suspension to 50 ml conical tube. **A gentle tap on the side of the tube, or brief vortexing, should dislodge pellet. At this point, the pellet and contents can be poured into the 50 ml conical tube**.

20. Add 5 ml of chloroform to the suspension, close tightly and vortex for 45 ± 15 sec to fully suspend the pellet.

21. Centrifuge at 1,700 x *g* for 15 min at 4 ± 2 °C.

22. Using a pipette, **carefully** collect the upper aqueous layer and transfer it to a clean ultracentrifuge tube - do not discard remaining sample. **Store ultracentrifuge tube containing aqueous layer on ice.**

23. Add 5 ml of Threonine solution to the remaining sample containing the chloroform interface fraction, close tightly, and vortex for 30 sec.

24. Centrifuge at 1,700 x *g* for 15 min at 4 ± 2 °C.

25. Using a pipette, **carefully** collect the top layer of the sample and combine with upper aqueous layer from step 22 into ultracentrifuge tube**.**

26. Discard remaining chloroform/sample as hazardous waste.

27. Add an additional 50 ml of t.c. PBS to sample in ultracentrifuge tube, bringing the total sample volume to ~ 60 ml).

28. Weigh and balance ultracentrifuge tubes with caps and contents to within 0.05 g of each other using t.c. PBS.

Note: This can be a stopping point in the protocol. Once the tubes are balanced and placed in the rotor with vacuum on, the samples can be left overnight at 4 ± 2 °C in the centrifuge and proceed with 1 hr spin the next day.

29. Centrifuge at 170,000 x g for 1 hr at 4 ± 2 °C.

30. Discard supernatant by pipetting **(pellet may be visible on side of tube or pellet may dislodge and fall to bottom).**

31. Let tube settle for 1 min and discard excess liquid with disposable micro-pipettor.

32. Add 400 µl of t.c. PBS to ultracentrifuge tube.

33. Resuspend pellet using a transfer pipette and evenly distribute into two clean DNase/RNase free microcentrifuge tubes.

34. Store these concentrates at -70 °C or proceed directly to RNA extraction.

Note: Only 1 tube is required for RNA extraction. The remaining tube should be stored at ≤-70 °C as reserve for future analysis.

RNA Extraction

Note: Before starting extraction, warm an aliquot of primer TE (Section A6) in 70 ºC heating block.

1. Obtain one tube of shellfish concentrate (if concentrate is frozen, allow thawing).
2. Add 500 µl 6 M GITC solution (Section A6).
3. Vortex 60 ± 30 sec to dissolve concentrate (typically takes 60-90 sec).
4. Add 700 µl of 50% EtOH (Section A6) and invert twice.
5. Pipette 700 µl of sample onto a RNeasy mini spin column.
6. Centrifuge 10,000 x g for 1 min at room temperature.
7. Place column in new collection tube and discard flow through.
8. Add remaining sample to column.
9. Centrifuge 10,000 x g for 1 min at room temperature.
10. Place column in new collection tube and discard flow through.
11. Add 700 µl RW1 buffer to spin column and incubate for 15 min at room temperature.

Note: Heat primer TE at 70 °C for at least 10 min prior to use.

12. Centrifuge at 10,000 x g for 1 min at room temperature.
13. Place column in new collection tube and discard flow through.
14. Add 500 µl RPE buffer to spin column and incubate for 15 min at room temperature.
15. Centrifuge at 10,000 x g for 1 min at room temperature.
16. Add additional 500 µl of RPE buffer to spin column.

Note: Incubation not required at this step.

17. Centrifuge at maximum speed (≥ 16,000 x g) for 2 min at room temperature.

18. Transfer column to new collection tube and centrifuge at room temperature full speed for 1 min to dry column.

19. Carefully transfer column to 1.5 ml low-retention/siliconized RNase/DNase free microcentrifuge tube.

20. Pipette 50 µL heated Primer TE buffer (Section A6) onto silica-gel membrane of column.

21. Centrifuge at 10,000 x g for 1 min at room temperature.

Note: Material that passed through column contains the viral RNA being extracted and is in the collection tube in which the cartridge was placed.

22. Pipette and additional 50 µL of heated Primer TE onto silica-gel membrane of column.

23. Pipette the eluted RNA back onto column (~50 µL).

24. Centrifuge at 10,000 x g for 1 min at room temperature.

25. Proceed with RT-qPCR or freeze RNA at ≤ -70 ºC for storage.

Section A4: Concentration and Extraction of Enteric Viruses from Scallops and Finfish

Note: For large, uncut finfish, aseptically remove sample from the surface to obtain 50 g.

1. Add 50 ± 2 g of scallops or fin fish (cut in no larger than 1" cubes pieces) to a Whirl-Pak plastic bag.

2. Add 100 µl extraction control virus (Section A5).

3. Add 50 ml of Glycine buffer, pH 9.5 (Section A6) and tightly close.

4. Shake at 200 rpm or medium speed for 15 min at room temperature.

5. Decant liquid into a 50 ml tube. Let bag sit for 2-3 min, shake side to side and pour remaining liquid into the 50 ml conical tube.

Note: Do not squeeze bag to obtain more buffer. This will cause PCR inhibition.

6. Centrifuge at 9,000 x *g* for 30 min at 4 ± 2 ºC.

7. Decant supernatant into a 70 ml ultracentrifuge tube.

8. Add 10 ml of t.c. PBS (Section A6) to each tube to bring volume up to ~60 ml.

9. Balance tubes to within 0.05 g of each other using t.c. PBS.

10. Centrifuge at 170,000 x *g* for 60 min at 4 ± 2 °C.

11. Carefully pipette and discard supernatant **(pellet may be visible on side of tube or pellet may dislodge and fall to bottom).**

Note: There may be a clear gelatinous substance; this will be resuspended in addition to the pellet.

12. Add 320 µl of t.c. PBS to the ultracentrifuge tube. Using a disposable transfer pipette, carefully resuspend sample. Transfer into one 2.0 ml DNase/RNase free tube.

13. Add 400 µl of chloroform to tube. Vortex for 40 ± 10 sec and spin at centrifuge at 2,000 x *g* for 15 min.

14. Evenly distribute aqueous layer into two separate 2.0 ml DNase/RNase free microcentrifuge tubes.

15. Store these concentrates at -70 °C until use or proceed directly to RNA extraction.

**** Note: Only 1 tube is required for RNA extraction. The remaining tube should be stored at -70 °C for future analysis***

RNA Extraction

Note: Before starting extraction, prepare AVL solution with carrier RNA and warm Buffer AVE in 70 ºC heating block.

1. Add 560 µl prepared Buffer AVL with carrier RNA (Section A6) to sample concentrate tube.

2. Incubate at room temperature (20-25 °C) for 10 min.

3. Resuspend pellet by pipetting up and down and vortexing.

4. Transfer 700 µl of liquid to the QIAshredder column.

5. Centrifuge 2 min at maximum speed (≥ 16,000 x *g*).

6. Carefully transfer the column flow-through fraction to a new 2.0 ml low retention/siliconized DNase/RNase free microcentrifuge tube without disturbing the cell-debris pellet (if present) in the collection tube. Discard collection tube.

7. Repeat steps 4-6 using another QIAshredder column, if necessary, until entire sample has been processed through the QIAshredder. Transfer the column flow through to the 2.0 ml micro-centrifuge tube in step 6.

8. Add 560 µl of 50% ethanol to the cleared lysate and mix immediately by pipetting. Do not centrifuge. Continue without delay to next step.

9. Apply 630 µl of the ethanol/lysate solution to a QIAamp mini column.

10. Centrifuge 8000 x *g* for 1 min. Place the QIAamp spin column in a new collection tube. Discard flow through and collection tube.

11. Repeat steps 9 and 10 until the entire sample has been passed through the column, discarding the collection tube each time.

12. Transfer the QIAamp mini column into a new 2 ml collection tube.

13. Add 500 µl Buffer AW1. Incubate for 10 min.

14. Centrifuge 1 min at 8,000 x *g*. Discard flow through and collection tube.

15. Transfer the QIAamp mini column into a new 2 ml collection tube.

16. Add 500 µl Buffer AW2 onto the QIAamp Mini column.

17. Centrifuge at maximum speed (≥ 16,000 x *g*) for 3 min. Discard flow through and collection tube.

18. Transfer the QIAamp mini column into a new 2 ml collection tube. Centrifuge at maximum speed (≥ 16,000 x g) for 1 min to dry column.

19. To elute RNA, transfer the QIAamp mini column in to a new 1.5 ml low retention/siliconized DNase/RNase free centrifuge tube.

20. Add 50 µl of pre-heated (70 ºC) Buffer AVE directly onto the QiaAmp silca-gel membrane.

21. Close the tube gently, and centrifuge for 1 min at 8,000 x *g*.

22. Pipet 50 µl of pre-heated Buffer AVE to column. Pipette the eluted 50 µl back to the top of the column.

23. Close the tube gently, and centrifuge for 1 min at 8,000 x *g*.

24. Discard column and place tube with RNA (step 23) on ice to prepare Zymo column.

25. Prepare Zymo column per manufactures instructions.

26. Transfer Zymo column into a clean 1.5 or 2.0 ml low-retention/siliconized RNase/DNase free microcentrifuge tube.

27. Transfer RNA from step 23 to prepared Zymo One Step RT-PCR inhibitor remover column.

28. Screw on cap loosely and spin at 8,000 x *g* for 3 min.

29. Proceed with RT-qPCR or freeze at -70 °C for storage.

Section A5: Extraction Control Preparation

Sample Extraction Control Preparation (Murine Norovirus and Mengovirus)

Note: The murine norovirus and mengovirus extraction control propagated in-house typically yields a titer of 10^5 PFU/ml. Murine norovirus and mengovirus purchased from ATCC or other commercial entity may have titers expressed as TCID50. The target concentration for the extraction control added to each sample will be ~10^3 PFU/g which will yield C_t's between 30-37. Instructions for appropriate dilutions of virus stocks in t.c. PBS with 20% glycerol is listed below.

1. Defrost thoroughly, on ice, extraction control virus stock solution and vortex for 5 sec.

2. Spin for 2 sec in a microcentrifuge to bring solution to bottom of tube.

3. Prepare one, 1:10 dilution blank for extraction control virus; 900 µl of t.c. PBS with 20% glycerol (Section A6).

4. Add 100 ul of extraction control stock solution to 900 µl dilution blank and vortex for 5 sec.

5. Spin for 2 sec in a microcentrifuge to bring solution to bottom of tube.

6. Aliquot in 110 µl volumes for future use. These aliquots are considered your diluted stocks of the extraction control.

Note: <u>Aliquots can be frozen for future matrix (sample)seeding, but titer will diminish after multiple freeze-thaw cycles.</u> A 100 µl aliquot of this dilution serves as the extraction control mentioned in the virus protocols. Please be mindful that the extraction control (MNV or mengovirus) is added to samples to determine if extractions were performed correctly <u>and not</u> to establish the sensitivity of the assay. This is a qualitative assay only.

Note: The optimal range for the detection of the diluted extraction control working stock is 30-37 C_ts. Due to degradation, the titer for can decrease overtime. When there is a trend where the extraction C_t averages are above 37 C_t with two consecutive runs and there is an absence of inhibition, a new aliquot of the diluted extraction control should be made and used as the extraction controls during sample analysis. Although the extraction control is not required to cross the threshold on or before 37 cycles for the run to be considered valid, this step is being recommended as a QA/QC step for maintaining optimal working stocks of the extraction control. All diluted working stocks of the extraction should be stored in a non-cycling (not frost free) -70 °C freezer to mitigate degradation.

Section A6: Buffers and Recipes

1. <u>t.c. PBS (tissue culture grade) with 20% glycerol</u>

10X PBS	10 ml
Sterile deionized water	90 ml

Obtain sterile container and add 80 ml of PBS and 20 ml volume of glycerol. Filter sterilize using a 0.22 µm filter. Store at 4 ± 2 °C

2. <u>Glycine Solution, pH 7.6</u>

NaCl	8.8 g
Glycine	56.3 g
Deionized water	800 ml

QS to make 1 L with deionized water. Adjust pH to 7.6, sterilize at 121°C for 15 min and store at 4 ± 2 °C.

3. <u>Primer TE (10 mM Tris, 0.1 mM EDTA, pH 8.0)</u>

1 M Tris pH 8.0	100 µl
0.05 M EDTA	20 µl
PCR-grade water(DNase/RNase free)	9.88 ml

Prepare in sterile 50 ml conical tube. Store at room temperature.

4. Carrier RNA solution

Buffer AVE	310 µl
310 µg carrier RNA	310 µg

Add 310 µl of buffer AVE to carrier RNA, store in 60 µl aliquots at -20 °C. If carrier RNA is different concentration, the ratio is 1:1 buffer AVE and carrier RNA.

5. AVL Buffer and AVE/Carrier RNA mix

Hydrate carrier RNA with buffer AVE (provided in QIAamp Viral RNA kit). Volumes of Buffer AVL and carrier RNA–Buffer AVE mix required for the number of extractions to be performed (refer to table below).

***Note: Store remaining carrier RNA in 30 µl aliquots at -20 °C.**

No. samples	Vol. Buffer AVL (ml)	Vol. carrier RNA–AVE (µl)	No. samples	Vol. Buffer AVL (ml)	Vol. carrier RNA–AVE (µl)
1	0.56	5.6	13	7.28	72.8
2	1.12	11.2	14	7.84	78.4
3	1.68	16.8	15	8.40	84.0
4	2.24	22.4	16	8.96	89.6
5	2.80	28.0	17	9.52	95.2
6	3.36	33.6	18	10.08	100.8
7	3.92	39.2	19	10.64	106.4
8	4.48	44.8	20	11.20	112.0
9	5.04	50.4	21	11.76	117.6
10	5.60	56.0	22	12.32	123.2
11	6.16	61.6	23	12.88	128.8
12	6.72	67.2	24	13.44	134.4

6. Threonine Buffer

DL-Threonine	59.6 g
NaCl	8.8 g

QS to make 1 L with deionized water. Adjust pH to 7.5, sterilize at 121 °C for 15 min and store at 4 ± 2 °C.

7. Glycine/Beef Extract Buffer

Glycine	3.75 g
Beef Extract	60.0 g
Tris Base	12.0 g
Distilled Water	800 ml H_20

QS with deionized water to make1 L. Adjust pH to 9.5, sterilize at 121 °C for 15 min and store at 4 ± 2 °C.

8. 2 M Potassium Acetate solution

Potassium Acetate	39.26 g
Distilled Water	150 ml

QS to 200 ml with deionized water. Sterilize at 121 °C for 15 min and store at room temperature.

9. Pectinase solution

10,000 U pectinase (*Aspergillus niger*)	1.25 g
DNase/RNase free PCR grade water	5 ml

Prepare and aliquot into dark 1.5 ml tubes, store at 4 ± 2 °C, discard after 6 months.

10. t.c. PBS

10X PBS (Sigma P5493)	100 ml
Sterile deionized water	900 ml

Store at 4 ± 2 °C.

Note: Can be used where protocols indicates t.c. PBS or formula below can be used

11. t.c. PBS

NaCl	8.0 g
KCl	0.2 g
KH_2PO_4	0.12 g
Na_2HPO_4	0.91 g

QS to make 1 L with deionized water. Adjust pH to 7.5, sterilize at 121 °C for 15 min and store at 4 ± 2 °C.

Note: Can be used where protocols indicates t.c. PBS or formula above can be used

12. **<u>6 M Guanidine Isothiocyanate (GITC)</u>**

GITC (Gibco/BRL 15535-016, 500 g)	7.09 g

Aseptically measure 7.09 grams of GITC into a sterile container. Add 4.5 ml of DNase/RNase free water to the container. Mix well until dissolved. A total volume of 10 ml of GITC solution should be noted.

Note: Store at room temperature in light-occluding DNase/RNase free tubes. Solution stable for 1 month.

13. **<u>5 M NaOH solution</u>**

NaOH	8.0 g

QS with sterile deionized water to 40 ml.

14. **<u>2.5 M NaOH solution</u>**

NaOH	4.0 g

QS with sterile deionized water to make 40 ml.

15. **<u>50% Ethanol</u>**

100% ethanol	5 ml

QS with sterile deionized water to make 10 ml. Store at room temperature.

16. **<u>Glycine -Tris solution, pH 9.5</u>**

Glycine (Sigma G-7126 or equivalent)	3.75 g
Tris Base	12.0 g
Deionized Water	800 ml H_20

QS to make 1 L with deionized water. Adjust pH to 9.5, sterilize at 121 °C for 15 min and store at 4 ± 2 °C.

SECTION B: VIRUS DETECTION

Note: Always wear gloves and never wear the same gloves to when going between master mix and samples. Assembly of master mix should be done in a clean master mix PCR hood or BSC hood that has been decontaminated with 10% Bleach solution or HypeWipes followed by 70% Ethanol, or similar product and UV irradiated for 20 min. Change gloves often and when exiting and/or reentering the hood. Always use aerosol resistant pipette tips for PCR.

Section B1: RT-qPCR Detection of Murine Norovirus on AB 7500 Platforms

RT-qPCR Assays

Outlined Murine Norovirus (MNV) RT-qPCR for detection murine norovirus on the AB7500. Primers, probes, and master mix preparation are found in Tables B1.1 and B1.2.

Sample Preparation - Murine Norovirus Protocol

Reverse transcription: 50 °C for 3000 sec (50 min)

Polymerase activation: 95 °C for 900 sec (15 min)

45 cycles of: 95 °C for 15 sec, 55 °C for 20 sec, 62 °C for 60 sec with optics on

Reaction Set-Up

Note: Always use aerosol resistant pipette tips for PCR.

1. Thaw primer solutions, probe solutions, and buffer mix and place them in 4 °C bench top cool block or on crushed ice in master mix set up hood.
2. Vortex reagents for 2-3 sec at setting 7-10, and then briefly centrifuge for 3-5 sec in a mini-centrifuge to settle the liquid to the bottom of the tube. Place in ice or bench top cooler. Keep Enzyme Mix in cooling block or on ice at all times, these enzymes should not be defrosted.
3. Prepare Master Mix for all sample and control reactions as listed in table B2.1 (additional master mix preparations can be found in Appendix E). Keep all thawed components, reagents, controls and master mixes in cooling block or on crushed ice.

***Note: Viral RNA templates should be added to reaction tubes in a designated area separate from location where master mixes are prepared. A negative and positive control should be added to each run set-up**.*

4. Proceed to hood/area or room where the template is added and thaw IAC RNA and sample RNA in the designated hood where the template is added. Briefly centrifuge the tubes 3-5 sec in micro-centrifuge (pulse spin) to settle the liquid at the bottom of the tube. Add appropriate volume of IAC, (0.2 µl/rxn) to Master Mix (keep cold); Vortex briefly & Pulse spin.

5. Add 22 µl Master Mix to each designated reaction tube or sample wells.

6. Add 3 µl of sample template to three designated reaction tubes or sample wells.

7. Close reaction tubes or seal sample plate once sample and appropriate controls have been added, briefly spin to mix bring down reagents.

Instrument Set-up

1. Open AB Software (version 2.0-2.3).

2. Fill out the "Experiment Name" field.

3. Select 7500 (FAST).

4. Select "Quantitation Standard Curve"

5. Select "TaqMan Reagents"

6. Select "Standard (~3 hours to complete run)"

7. Click "Plate Set Up"

8. Under the "Define Targets" area click on the "Add New Target" until you have 2 targets.

9. Fill Target 1 with MNV, Target 2 with IAC.

10. Change the reporter dye to Cy5 for MNV and TexRd for IAC (do not change quenchers from the default NFQ- MGB).

11. Under Sample section click "Add Samples" until you have the requisite number of samples.

12. Fill in your sample name in each of the areas provided.

13. Click “Assign Targets & Samples” tab and name samples and assign samples to the appropriate well(s).
14. Select FAM as the passive reference dye.
15. Select “Run Method”
16. The first holding stage is 3000 sec (50 min) at 50 °C.
17. The second holding stage is 900 sec (15 min) at 95 °C.
18. Cycling stage repeats 45 cycles.
19. First stage 15 sec at 95 °C.
20. Second stage 20 sec at 55 °C.
21. Third stage set at 60 sec (1 min) at 62 °C (click the icon for collecting data at “collect data on hold”).
22. Save the run method – Save as MNV Multiplex.
23. Click the “Analysis” tab on the right.
24. In the upper right corner click on “Analysis Settings.”
25. For “Data Analysis change settings for Cy5 (MNV) and TexRd (Internal Control): Threshold set to 0.1 and “Baseline Start Cycle” to 3 and “End Cycle” to 10.
26. Click “Apply Analysis Settings.”
27. Click “Run” tab on the right.
28. Click green “Start Run” box.

Data Interpretation - Murine Norovirus Multiplex Assay

1. Repeat any invalid sample. Sample is “invalid” if:
    a. The RT-qPCR negative control demonstrates positive C_t results for MNV in Cy5 or if the IAC is negative (no C_t from TxRed),
    b. The RT-qPCR positive control is negative (no C_t from Cy5) for MNV,
    c. The MNV RT-qPCR is negative (no C_t from Cy5) for any sample,
    d. The average of the IAC C_t values for the sample replicates are more than 4.0 C_t’s greater than the negative control IAC C_t value, repeat the RT-

qPCR assay using remaining RNA or RNA from a newly extracted tube with a 1 µl template in the RT-qPCR reaction in triplicate. If the 1 µl template reactions yields an average IAC C_t values greater than 4.0 C_t higher than the Negative Control IAC C_t value, repeat the sample analysis from the beginning using additional food sample. With the new sample, the concentrates will be split into 5 tubes and complete RT-qPCR with 1 µl reactions in triplicate.

2. Sample is "valid" and can be reported if:

   a. RT-qPCR negative control is negative for MNV,

   b. RT-qPCR positive control is positive for MNV,

   c. RT-qPCR is positive for MNV in all spiked matrices,

   d. Internal amplification control (IAC) is positive in all reactions and average of the IAC C_t values for sample is within 4.0 C_t's of the negative control IAC C_t value.

Note: For MNV, if the average of the IAC C_t values for the sample replicates are more than 4.0 C_t's greater than the Negative Control IAC C_t value AND the corresponding sample is positive for norovirus and/or hepatitis A virus, the MNV RTqPCR does not have to be repeated. If norovirus or hepatitis A virus is detected in a sample that has inhibition present in the RTqPCR reaction and has log amplification, this sample does not need to be repeated for norovirus or hepatitis A virus RTqPCR and would be considered positive. Repeating RT-qPCR reactions due to inhibition is to ensure that you do not have false negatives.

Tables

Table B1.1 Primer and Probe Sequences for MNV and Internal Amplification Control

Identification	**Primers**	**Location[c]**
MNVR[2]	5’ CAC AGA GGC CAA TTG GTA AA 3’	6645-6626
MNVF	5’- TGC AAG CTC TAC AAC GAA GG -3’	6520-6539
IC46F[a]	5’GAC ATC GAT ATG GGT GCC G-3’	N/A
IC194R[a]	5’-AAT ATT CGC GAG ACG ATG CAG-3’	N/A
MNVP	Cy5- 5’ CCT TCC CGA CCG ATG GCA TC 3’-IB-RQ[b]	6578-6594
IACP	TxR –5’ TCT CAT GCG TCT CCC TGG TGA ATG TG-IB RQ 3’ *	N/A

[2]Hewitt, Rivera-Aban, Greening 2009

[a] Depaola, Jones, Woods et. al. 2010. Internal Amplification Control (IAC) primers and probes are covered by U.S. Patent Application 0060166232.
[b]IB RQ- Iowa Black RQ
[c]based on accession no. JF320650

Table B1.2 AB 7500 Amplification Reaction Components and Master Mix Volume for MNV

Reagent	**Initial Concentration**	**Volume per 25 µl reaction**	**Final Concentration**
RNase Free H_20		9.8 µl	-
5X OneStep RT-PCR Buffer	5X	5.0 µl	1X
$MgCl_2$~	50 mM	0.75 µl	1.5 mM
dNTP Mix	10 mM	1 µl	0.4 mM
MNVF	10 µM	0.50 µl	0.2 µM
MNVR	10 µM	0.50 µl	0.2 µM
IC 46F	10 µM	0.1875 µl	0.075 µM
IC 194R	10 µM	0.1875 µl	0.075 µM
MNVP	10 µM	0.25 µl	0.1 µM
IACP	10 µM	0.375 µl	0.15 µM
OneStep RT- qPCR Enzyme Mix		1.00 µl	
Superasin	20 Units/µl	0.25 µl	5 Units
FAM ref dye	500 nM	2 µl	0.04 µM
Internal Amplification Control RNA		*0.2 µl	-
RNA		3 µl	

*Amount varies with concentration of IAC RNA. The amount of IAC template needs to be adjusted based on the prepared stock concentration to report Cycle threshold (Ct) of 20-25 when no inhibition is present in the reaction. The required concentration was provided to each laboratory participating in the validation study.

˜With the addition of 1.5 mM $MgCl_2$ the final concentration per reaction is 4.0 mM MgCl2

Section B2: RT-qPCR Detection of Hepatitis A Virus on the AB 7500 Platform

RT-qPCR Assay

Outlined Hepatitis A Virus RT-qPCR for detection hepatitis A virus on the AB7500. Primers, probes, and master mix preparation are found in Tables B2.1 and B2.2.

Sample Preparation - Hepatitis A Virus Thermo-cycling Protocol

Reverse transcription: 50 °C for 3000 sec (50 min)

Polymerase activation: 95 °C for 900 sec (15 min)

50 cycles of: 95 °C for 10 sec, 53 °C for 25 sec, 64 °C for 70 sec with optics on

Reaction Set-Up

***Note: Always use aerosol resistant pipette tips for PCR.**

1. Thaw primer solutions, probe solutions, and buffer mix and place them in 4 °C bench top cool block or on ice in master mix set up hood.
2. Vortex reagents for 2-3 sec at setting 7-10, and then briefly centrifuge for 3-5 sec in a mini-centrifuge to settle the liquid to the bottom of the tube. Place in ice or bench top cooler. Keep Enzyme Mix in cooling block or on ice at all times, these enzymes should not be defrosted.
3. Prepare Master Mix for all sample and control reactions as listed in table B2.2 (additional master mix preparations can be found in Appendix F. Keep all thawed components, reagents, controls and master mixes in cooling block.

***Note: Viral RNA templates should be added to reaction tubes in a designated area separate from location where master mixes are prepared. negative and positive controls should be added to each reaction set-up.**

4. Proceed to hood/area or room where the template is added and thaw IAC RNA and sample RNA in the designated hood where the template is added. Briefly centrifuge the tubes 3-5 sec in micro-centrifuge to settle the liquid at the bottom of the tube. Add appropriate volume of IAC, (0.2 µl/rxn) to Master Mix (keep cold); Vortex briefly & Pulse spin.
5. Add 22 µl Master Mix to each designated reaction tube or sample wells.

6. Add 3 µl of sample template to three designated reaction tubes or sample wells.

7. Close reaction tubes or seal sample plate once sample and appropriate controls have been added, briefly spin to mix bring down reagents.

Instrument Set-Up

1. Open AB Software (version 2.0-2.3).
2. Fill out the “Experiment Name” field.
3. Select 7500 (FAST).
4. Select “Quantitation Standard Curve”
5. Select “TaqMan Reagents”
6. Select “Standard (~3 hours to complete run)”
7. Click “Plate Set Up”
8. Under the “Define Targets” area click on the “Add New Target” until you have 2 targets.
9. Fill Target 1 with HAV, Target 2 with IAC.
10. Change the reporter dye to Cy5 for MNV and TexRd for IAC (do not change quenchers from the default NFQ- MGB).
11. Under Sample section click “Add Samples” until you have the requisite number of samples.
12. Fill in your sample name in each of the areas providedl
13. Click “Assign Targets & Samples” tab and name samples and assign samples to the appropriate well(s).
14. Select FAM as the passive reference dye.
15. Select “Run Method”
16. The first holding stage is 3000 sec (50 min) at 50 °C.
17. The second holding stage is 900 sec (15 min) at 95 °C.
18. Cycling stage repeats 50 cycles.
19. First stage 10 sec at 95 °C.

20. Second stage 25 sec at 53 °C.

21. Third stage set at 70 sec (1 min 10 sec) at 64 °C (click the icon for collecting data at “collect data on hold”).

22. Save the run method – Save as HAV Multiplex.

23. Click the “Analysis” tab on the right.

24. In the upper right corner click on “Analysis Settings”

25. For “Data Analysis change settings for Cy5 (HAV) and TexRd (Internal Control): Threshold set to 0.1 and “Baseline Start Cycle” to 3 and “End Cycle” to 10.

26. Click “Apply Analysis Settings”

27. Click “Run” tab on the right.

28. Click green “Start Run” box.

Data Interpretation - HAV Multiplex Assay

For this HAV multiplex assay, Cy5 is the HAV probe fluorescent label and Texas Red (TxR) is the internal amplification control (IAC) probe fluorescent label.

1. Sample is “negative" if:
    a. RT-qPCR negative control is negative for HAV,
    b. RT-qPCR positive control is positive for HAV,
    c. Matrix control sample (if included) is negative for HAV,
    d. Unknown is negative for HAV,
    e. Internal amplification control (IAC) is positive. No further analysis is needed.
2. Sample is “positive” if:
    a. RT-qPCR negative control is negative for HAV,
    b. RT-qPCR positive control is positive for HAV,
    c. Unknown sample is positive for the detection HAV.
3. Samples are invalid if:
    a. If the negative RT-qPCR control sample demonstrates positive results

crossing the Cy5 or if the IAC is negative, repeat the RTqPCR assay,

a. The average of the IAC C_t values for the sample replicates are more than 4.0 C_t's greater than the Negative Control IAC C_t value, repeat the RT-qPCR assay using remaining RNA or RNA from a newly extracted tube with a 1 µl RT-qPCR reaction in triplicate. If the repeat of the newly extracted sample yields average IAC C_t values 4.0 C_t's higher than the Negative Control IAC C_t value, repeat the sample analysis from the beginning using additional food. With the new repeat, the concentrates will be split into 5 tubes and complete RT-qPCR with 1µl reactions in triplicate.

Note: A positive sample is a result that demonstrates log amplification. Log amplification can be viewed as a graph on the ABI 7500 Fast platform. If the sample does not exhibit log amplification and crosses the threshold, the RTqPCR reaction should be repeated.

Tables

Table B2.1. Primer and Probe Sequences for HAV and Internal Amplification Control RNA

Identification	**Primers**	**Location** [c]
GAR2F	5' ATA GGG TAA CAG CGG CGG ATA T 3'	448-469
GAR1R	5'-CTC AAT GCA TCC ACT GGA TGA G-3'	517-537
IC46F[a,b]	5'GAC ATC GAT ATG GGT GCC G-3'	N/A
IC194R[a,b]	5'-AAT ATT CGC GAG ACG ATG CAG-3'	N/A
	Probes	
GARP	Cy5- 5' AGA CAA AAA CCA TTC AAC GCC GGA GG 3' -IB-RQ*	483-508
IACP[a,b]	TxR –TCT CAT GCG TCT CCC TGG TGA ATG TG -IB RQ*	N/A

[a]Internal Amplification Control (IAC) primers and probes are covered by U.S. Patent Application 0060166232
[b]Depaola, Jones, Woods, et al. 2010.
[c]Based on GenBank accession # M14707

*IB RQ- Iowa Black RQ
*Amount varies with concentration of IAC RNA. The amount of IAC template needs to be adjusted based on the prepared stock concentration to report Cycle threshold (Ct) at about 20-25 when no inhibition is present in the reaction. The required concentration was provided to each participating laboratory.

˜With the addition of 1.5 mM $MgCl_2$, the final concentration per reaction is 4.0 mM $MgCl_2$

Table B2.2. AB 7500 Amplification Reaction Components and Master Mix Volume for HAV

Reagent	**Initial Concentration**	**Volume per 25 µl reaction**	**Final Concentration**
RNase Free H_20		9.05 µl	-
5X OneStep RT-PCR Buffer	5X	5.0 µl	1X
$MgCl_2$~	50 mM	0.75 µl	1.5 mM
dNTP Mix	10 mM	1 µl	0.4 mM
GAR2F	10 µM	0.75 µl	0.3 µM
GAR1R	10 µM	0.75 µl	0.3 µM
IC 46F	10 µM	0.1875 µl	0.075 µM
IC 194R	10 µM	0.1875 µl	0.075 µM
GARP	10 µM	0.5 µl	0.2 µM
IACP	10 µM	0.375 µl	0.15 µM
FAM ref dye	500 nM	2 µl	0.04 µM
OneStep RT-PCR Enzyme Mix		1.00 µl	
Superase·in	20 Units/µl	0.25 µl	5 Units
Internal Amplification Control RNA		*0.2 µl	-
RNA		3 µl	

*Amount varies with concentration of IAC RNA. The amount of IAC template needs to be adjusted based on the prepared stock concentration to report Cycle threshold (Ct) at about 20-25 when no inhibition is present in the reaction. The required concentration was provided to each participating laboratory.

˜With the addition of 1.5 mM $MgCl_2$, the final concentration per reaction is 4.0 mM $MgCl_2$

Section B3: RT-qPCR Detection of Hepatitis A Virus Control Exclusion Assay on the AB 7500 Platform

RT-qPCR Assays

Outlined HAV Control Exclusion Assay for AB 7500 (Primers, probes, and master mix preparation are found in Table B3.1 and B3.2.

****Note: If a 1 µl sample volume was used for the initial HAV RT-qPCR assay due to inhibition, you will use a 1 µl sample volume for this assay. Adjust master mix volumes accordingly.***

Sample Preparation - HAV Control Exclusion Assay

Reverse transcription: 50 °C for 3000 sec

Polymerase activation: 95 °C for 900 sec

50 cycles of: 95 °C for 10 sec, 54 °C for 25 sec, 64 °C for 60 sec with optics on

Reaction Set-Up

****Note: Always use aerosol resistant pipette tips for PCR.***

1. Thaw primer solutions, probe solutions, and buffer mix and place them in 4 °C bench top cool block or on ice in master mix set up hood.
2. Vortex reagents for 2-3 sec at setting 7-10, and then briefly centrifuge for 3-5 sec in a mini-centrifuge to settle the liquid to the bottom of the tube. Place in ice or bench top cooler. Keep Enzyme Mix in cooling block or on ice at all times, these enzymes should not be defrosted.
3. Prepare Master Mix for all sample and control reactions as listed in table B3.1 (additional master mix preparations can be found in Appendix F). Keep all thawed components, reagents, controls and master mixes **in cooling block or on ice**.

****Note: Viral RNA templates should be added to reaction tubes in a designated area separate from location where master mixes are prepared. A negative and positive control should be added to each run set-up.***

4. Proceed to hood/area or room where the template is added and thaw IAC RNA and sample RNA in the designated hood where the template is added. Briefly centrifuge the tubes 3-5 sec in micro-centrifuge to settle the liquid at the bottom

of the tube. Add appropriate volume of IAC, (0.2 µl/rxn) to Master Mix (keep cold); Vortex briefly & Pulse spin.

5. Add 22 µl Master Mix to each designated reaction tube or sample wells.

6. Add 3 µl of sample template to two designated reaction tubes or sample wells.

7. Close reaction tubes or seal sample plate once sample and appropriate controls have been added, briefly spin to mix bring down reagents.

Instrument Set-up

1. Open AB Software (version 2.0-2.3).

2. Fill out the "Experiment Name" field.

3. Select 7500 (FAST).

4. Select "Quantitation Standard Curve"

5. Select "TaqMan Reagents"

6. Select "Standard (~3 hours to complete run)"

7. Click "Plate Set Up"

8. Under the "Define Targets" area click on the "Add New Target" until you have 2 targets.

9. Fill Target 1 with CEA, Target 2 with IAC.

10. Change the reporter dye to Cy5 for CEA and TexRd for IAC (do not change quenchers from the default NFQ- MGB).

11. Under Sample section click "Add Samples" until you have the requisite number of samples.

12. Fill in your sample name in each of the areas provided.

13. Click "Assign Targets & Samples" tab and name samples and assign samples to the appropriate well(s).

14. Select FAM as the passive reference dye.

15. Select "Run Method"

16. The first holding stage is 3000 sec (50 min) at 50 °C.

17. The second holding stage is 900 sec (15 min) at 95 °C.

18. Cycling stage repeats 50 cycles.

19. First stage 10 sec at 95 °C.

20. Second stage 25 sec at 54 °C.

21. Third stage set at 60 sec (1 min) at 64 °C (click the icon for collecting data at "collect data on hold").

22. Save the run method – Save as CEA Multiplex.

23. Click the "Analysis" tab on the right.

24. In the upper right corner click on "Analysis Settings"

25. For "Data Analysis change settings Use Default Settings", "Automatic Threshold" and "Automatic Baseline" settings.

26. Click "Run" tab on the right.

27. Click green "Start Run" box.

Data Interpretation - Control Exclusion Assay

1. Any sample CEA RT-qPCR assay which negative for Cy5 (0 C_t value) and all controls are satisfactory, the virus detected in the HAV RT-qPCR assay was not the laboratory strain.

Note: If there was inhibition in the HAV RT-qPCR, this will be reflected in the internal amplification control for this assay also.

2. Any sample CEA RT-qPCR reaction which is positive for Cy5 and all controls are satisfactory will be considered a 'cannot rule out' and will require gel analysis with 3% agarose or genetic bioanalyzer for confirmation of the presence or absence of the wild type strain. See Appendix F.

3. If the RT-qPCR negative control demonstrates positive C_t results for HAV in Cy5, if the RT-qPCR positive control is negative (no C_t from Cy5) for HAV, or if the IAC is negative (no C_t from TxRed) the results are invalid; repeat assay due to nvalid results.

Tables

Table B3.1. Primer and Probe Sequences for HAV CEA

Identification	**Primers**	**Location** [∞]
HAVCROF	5’ CCGTTTGCCTAGGCTATAGGCT 3’	45-66
JWCROR	5’- GGA GAG CCC TGG AAG AAA GAA GA -3’	202-225
IC46F	5’GAC ATC GAT ATG GGT GCC G-3’	N/A
IC194R	5’-AAT ATT CGC GAG ACG ATG CAG-3’	N/A
	Probes	
JWCROP	Cy5 (TAO)-TGATTTGTAAATATTGATTCCTGCAG -IB-RQ	122-147
IACP	TxR –TCT CAT GCG TCT CCC TGG TGA ATG TG -IB RQ*	N/A

[∞]based on GenBank accession # M59808

Table B3.2. Amplification Reaction Components

Reagent	**Initial Concentration**	**Volume per 25 µl reaction**	**Final Concentration**
RNase Free H_20		8.425 µl	
5X OneStep RT-PCR Buffer	5X	5.0 µl	1X
*$MgCl_2$	25 mM	3 µl	1.5 mM
dNTP Mix	10 mM	0.75 µl	0.3 mM
CROF	10 µM	0.1875 µl	0.075 µM
JWCROR	10 µM	0.1875 µl	0.075 µM
IC 46F[b,c]	10 µM	0.1875 µl	0.075 µM
IC 194R[b,c]	10 µM	0.1875 µl	0.075 µM
JWCRO Probe	10 µM	0.25 µl	0.1 µM
IAC Probe	10 µM	0.375 µl	0.15 µM
FAM Ref Dye	500 nM	2 µl	0.04 µM
Superase·in	5 Units/µl	0.25 µl	1.25 Units
Enzyme Mix		1.00 µl	
Internal Control RNA[∞]		0.2 µl	
Template		3 µl[β]	

*for 50 mM concentration this will be 1.5 µl per reaction and 11.925 H_2O

[b]Internal Amplification Control (IAC) primers and probes are covered by U.S. Patent Application 0060166232

[c]Depaola, Jones, Woods, et al. 2010.

[β]If the 1 µl template was used due to inhibition, the same template volume will be used for this assay. The volume of H_2O will be 10.425 per reaction.

Section B4: RT-qPCR Detection of Norovirus GI and GII Assay on the AB 7500 Platform

RT-qPCR Assay

Outlined Norovirus RT-qPCR for detection of norovirus GI and GII AB 7500 (Primers, probes, and master mix preparation are found in Tables B4.1 and B4.2).

Sample Preparation - Norovirus Thermal Cycling Protocol

Reverse transcription: 50 °C for 3000 sec (50 min)

Polymerase activation: 95 °C for 900 sec (15 min)

50 cycles of: 95 °C for 10 sec, 53 °C for 25 sec, 62 °C for 70 sec with optics on

Reaction Set-Up

***Note: Always use aerosol resistant pipette tips for PCR.**

1. Thaw primer solutions, probe solutions, and buffer mix and place them in 4 °C bench top cool block or on ice in master mix set up hood.

2. Vortex reagents for 2-3 sec at setting 7-10, and then briefly centrifuge for 3-5 sec in a mini-centrifuge to settle the liquid to the bottom of the tube. Place in ice or bench top cooler. Keep Enzyme Mix in cooling block or on ice at all times, these enzymes should not be defrosted.

3. Prepare Master Mix for all sample and control reactions as listed in table B2.1 (additional master mix preparations can be found in Appendix G). Keep all thawed components, reagents, controls and master mixes in cooling block or on ice.

***Note: Viral RNA templates should be added to reaction tubes in a designated area separate from location where master mixes are prepared. A negative and positive control should be added to each run set-up.**

6. Proceed to hood/area or room where the template is added and thaw IAC RNA and sample RNA in the designated hood where the template is added. Briefly centrifuge the tubes 3-5 sec in micro-centrifuge to settle the liquid at the bottom of the tube. Add appropriate volume of IAC, (0.2 µl/rxn) to Master Mix (keep cold); Vortex briefly & Pulse spin.

7. Add 22 µl Master Mix to each designated reaction tube or sample wells.
8. Add 3 µl of sample template to three designated reaction tubes or sample wells.
9. Seal sample plate once sample and appropriate controls have been added, briefly spin to mix bring down reagents.

Instrument Set-up

1. Open AB Software (version 2.0-2.3).
2. Fill out the “Experiment Name” field.
3. Select 7500 (FAST).
4. Select “Quantitation Standard Curve”
5. Select “TaqMan Reagents”
6. Select “Standard (~3 hours to complete run)”
7. Click “Plate Set Up”
8. Under the “Define Targets” area click on the “Add New Target” until you have 2 targets.
9. Fill Target 1 with NoV GI, fill target 2 with NoV GII and fill Target 3 with IAC.
10. Change the reporter dye to Cy5 for NoV GI, Cy3 for NoV GII, and TexRd for IAC (do not change quenchers from the default NFQ- MGB).
11. Under Sample section click “Add Samples” until you have the requisite number of samples.
12. Fill in your sample name in each of the areas provided.
13. Click “Assign Targets & Samples” tab and name samples and assign samples to the appropriate well(s).
14. Select FAM as the passive reference dye.
15. Select “Run Method”
16. The first holding stage is 3000 sec (50 min) at 50 °C.
17. The second holding stage is 900 sec (15 min) at 95 °C.
18. Cycling stage repeats 50 cycles.

19. First stage 10 sec at 95 °C.

20. Second stage 25 sec at 53 °C.

21. Third stage set at 70 sec (1 minute 10 sec) at 62 °C (click the icon for collecting data at “collect data on hold”).

22. Save the run method – Save as Norovirus Multiplex.

23. Click the “Analysis” tab on the right.

24. In the upper right corner click on “Analysis Settings”

25. For “Data Analysis change settings for Cy5 (NoV GI) and TexRd (Internal Control) set threshold set to 0.1, for Cy3 (NoV GII) set threshold to 0.01. Set “Baseline Start Cycle” to 3 and “End Cycle” to 10.

26. Click “Apply Analysis Settings”

27. Click “Run” tab on the right.

28. Click green “Start Run” box.

Data Interpretation - Norovirus Multiplex Assay

For this NoV multiplex assay, Cy5 is the GI probe fluorescent label, Cy3 is the GII probe fluorescent label, and that Texas Red (TxR) is the internal amplification control (IAC) probe fluorescent label.

2. Sample is “negative" if:

    a. RT-qPCR negative control is negative for GI and GII,

    b. RT-qPCR positive control is positive for GI and GII,

    c. Matrix control sample (if included) is negative for GI and GII,

    d. Unknown is negative for GI and GII,

    e. Internal amplification control (IAC) is positive. No further analysis is needed.

3. Sample is “positive” if:

    a. RT-qPCR negative control is negative for GI and GII,

    b. RT-qPCR positive control is positive for GI and GII,

c. Unknown sample is positive for GI and/or GII.

4. Samples are invalid if:

a. If the negative RT-qPCR control sample demonstrates positive results crossing the Cy5 or Cy3 threshold or if the IAC is negative, repeat the RT-qPCR assay,

b. The RT-qPCR positive control is negative for GI and/or GII,

c. The average of the IAC C_t values for the sample replicates are more than 4.0 C_ts greater than the Negative Control IAC C_t value, repeat the RT-qPCR assay using remaining RNA or RNA from a newly extracted saved tube with a 1 µl RT-qPCR reaction in triplicate. If the repeat of the newly extracted sample yields average IAC C_t values 4.0 C_t 's higher than the Negative Control IAC C_t value, repeat the sample analysis from the beginning using additional food sample. With the new repeat, the concentrates will be split into 5 tubes and complete RT-qPCR with 1µl reactions in triplicate.

Note: A positive sample is a result that demonstrates log amplification. Log amplification can be viewed as a graph on the ABI 7500 Fast platform. If the sample does not exhibit log amplification and crosses the threshold, the RTqPCR reaction should be repeated.

Tables

Table B4.1. Primer and Probe Sequences for Norovirus and Internal Amplification Control RNA

Identification	**Primers**	**Location**[∞]
COG1R[a,∞]	5’ CTT AGA CGC CAT CAT CAT TYA C 3’	5350-5371
COG2R[a,β]	5’ TCG ACG CCA TCT TCA TTC ACA 3’	5080-5100
COG1F[a,∞]	5’ CGY TGG ATG CGN TTY CAT GA 3’	5287-5306
COG2F[a,β]	5’ CAR GAR BCN ATG TTY AGR TGG ATG AG 3’	5003-5028
IC46F[b,c]	5’GAC ATC GAT ATG GGT GCC G-3’	N/A
IC194R[b,c]	5’-AAT ATT CGC GAG ACG ATG CAG-3’	N/A
	Probes	
COGP[a,∞]	Cy5- 5’ (TAO) AGA TYG CGA TCY CCT GTC CA 3’ -IB-RQ*	5317-5336
COGP1b[a,∞]	Cy5- 5’ (TAO) AGA TCG CGG TCT CCT GTC CA 3’ -IB-RQ*	5317-5336
COG2P[a,β]	Cy3- 5’ TGG GAG GGC GAT CGC AAT CT 3’ -IB-RQ*	5048-5067
IACP[b,c]	TxR –TCT CAT GCG TCT CCC TGG TGA ATG TG -IB RQ*	N/A

[a]Kageyama et al., 2003,
[b]Internal Amplification Control (IAC) primers and probes are covered by U.S. Patent Application 0060166232
[c]Depaola, Jones, Woods, et al. 2010.

[∞]Based on GenBank accession # KF039728
[β]Based on GenBank accession # EF684915

*IB RQ- Iowa Black RQ

Table B4.2. ABI 7500 Amplification Reaction Components for Norovirus

Reagent	Initial Concentration	Volume per 25 µl reaction	Final Concentration
RNase Free H20		7.3 µl	-
5X OneStep RT-PCR Buffer	5X	5.0 µl	1X
$MgCl_2$~	50 mM	0.75 µl	1.5 mM
dNTP Mix	10 mM	1 µl	0.4 mM
COG1F	10 µM	0.75 µl	0.3 µM
COG1R	10 µM	0.75 µl	0.3 µM
COG2F	10 µM	0.75 µl	0.3 µM
COG2R	10 µM	0.75 µl	0.3 µM
IC 46F	10 µM	0.1875 µl	0.075 µM
IC 194R	10 µM	0.1875 µl	0.075 µM
COG1P	10 µM	0.25 µl	0.1 µM
COG1Pb	10 µM	0.25 µl	0.1 µM
COG2P	10 µM	0.25 µl	0.1 µM
IACP	10 µM	0.375 µl	0.15 µM
OneStep RT-PCR Enzyme Mix		1.00 µl	
Superase·in	20 Units/µl	0.25 µl	5 Units
FAM ref dye	500 nM	2 µl	0.04 µM
Internal Amplification Control RNA		*0.2 µl	-
RNA		3 µl	

*Amount varies with concentration of IAC RNA. The amount of IAC template needs to be adjusted based on the prepared stock concentration to report Cycle threshold (C_t) of 20-25 when no inhibition is present in the reaction. The required concentration was provided to each laboratory participating in the validation study.

˜With the addition of 1.5 mM MgCl, the final concentration per reaction is 4.0 mM MgCl.

Section B5: RT-qPCR Detection of Mengovirus on ABI 7500 Platforms

RT-qPCR Assays

Outlined Mengovirus RT-qPCR Assay for AB 7500 (Primers, probes, and master mix preparation are found in Table B5.1 and B5.2.

Sample Preparation – Mengovirus

Reverse transcription: 50 °C for 3000 sec (50 min)

Polymerase activation: 95 °C for 900 sec (15 min)

Cycling: 45 cycles of 95 °C for 10 sec, 50 °C for 25 sec, 62 °C for 60 sec with optics on

Reaction Set-Up

Note: Always use aerosol resistant pipette tips for PCR.

1. Thaw primer solutions, probe solutions, and buffer mix and place them in 4 °C bench top cool block or on ice in master mix set up hood.
2. Vortex reagents for 2-3 sec at setting 7-10, and then briefly centrifuge for 3-5 sec in a mini-centrifuge to settle the liquid to the bottom of the tube. Place in ice or bench top cooler. Keep Enzyme Mix in cooling block or on ice at all times, these enzymes should not be defrosted.
3. Prepare Master Mix for all sample and control reactions as listed in table B5.1 (additional master mix preparations can be found in Appendix H). Keep all thawed components, reagents, controls and master mixes in cooling block or on ice.

Note: Viral RNA templates should be added to reaction tubes in a designated area separate from location where master mixes are prepared. A negative and positive control should be added to each run set-up.

4. Proceed to hood/area or room where the template is added and thaw IAC RNA and sample RNA in the designated hood where the template is added. Briefly centrifuge the tubes 3-5 sec in micro-centrifuge to settle the liquid at the bottom of the tube. Add appropriate volume of IAC, (0.2 µl/rxn) to Master Mix (keep cold); Vortex briefly & Pulse spin.
5. Add 22 µl Master Mix to each designated reaction tube or sample wells.

6. Add 3 µl of sample template to three designated reaction tubes or sample wells.

7. Close reaction tubes or seal sample plate once sample and appropriate controls have been added, briefly spin to mix bring down reagents.

Instrument Set-up

1. Open AB Software (version 2.0-2.3).
2. Fill out the “Experiment Name” field.
3. Select 7500 (FAST).
4. Select “Quantitation Standard Curve”
5. Select “TaqMan Reagents”
6. Select “Standard (~3 hours to complete run)”
7. Click “Plate Set Up”
8. Under the “Define Targets” area click on the “Add New Target” until you have 2 targets.
9. Fill Target 1 with Mengovirus, Target 2 with IAC.
10. Change the reporter dye to Cy5 for Mengovirus and TexRd for IAC (do not change quenchers from the default NFQ- MGB).
11. Under Sample section click “Add Samples” until you have the requisite number of samples.
12. Fill in your sample name in each of the areas provided.
13. Click “Assign Targets & Samples” tab and name samples and assign samples to the appropriate well(s).
14. Select FAM as the passive reference dye.
15. Select “Run Method”
16. The first holding stage is 3000 sec (50 min) at 50 °C.
17. The second holding stage is 900 sec (15 min) at 95 °C.
18. Cycling stage repeats 45 cycles.
19. First stage 10 sec at 95 °C.

20. Second stage 25 sec at 50 °C.

21. Third stage set at 60 sec (1 min) at 62 °C (click the icon for collecting data at "collect data on hold").

22. Save the run method – Save as Mengovirus Multiplex.

23. Click the "Analysis" tab on the right.

24. In the upper right corner click on "Analysis Settings"

25. For "Data Analysis change settings for Cy5 (Mengovirus) and TexRd (Internal Control): Threshold set to 0.1 and "Baseline Start Cycle" to 3 and "End Cycle" to 10.

26. Click "Apply Analysis Settings"

27. Click "Run" tab on the right.

28. Click green "Start Run" box.

Data Interpretation - Mengovirus Assay

For this Mengovirus multiplex assay, Cy5 is the Mengovirus probe fluorescent label and Texas Red (TxR) is the internal amplification control (IAC) probe fluorescent label.

1. Repeat any "invalid" samples. Sample is "invalid" if:

    a. The negative RT-qPCR control sample demonstrates positive results crossing the Cy5 or if the IAC is negative,

    b. The RT-qPCR positive control is negative for Mengovirus,

    c. The Mengovirus RT-qPCR is negative in any sample,

    d. The average of the IAC C_t values for the sample replicates are more than 4.0 C_ts greater than the Negative Control IAC C_t value, repeat the RT-qPCR assay using remaining RNA or RNA from a newly extracted tube with a 1 µl template in the RT-qPCR reaction in triplicate. If the 1 µl template reactions yields an average IAC C_t values greater than 4.0 C_t higher than the Negative Control IAC C_t value, repeat the sample analysis from the beginning using additional food sample. With the new sample, the concentrates will be split into 5 tubes and complete RT-qPCR with 1 µl reactions in triplicate.

2. Sample is "valid" and can be reported if:

    a. RT-qPCR negative control is negative for Mengovirus,

    b. RT-qPCR positive control is positive for Mengovirus,

    c. RT-qPCR is positive for Mengovirus in all spikes matrices,

    d. Internal amplification control (IAC) is positive in all reactions and average of the IAC C_t values for sample is within 4.0 C_ts of the Negative Control IAC C_t Value.

Note: For mengovirus, if the average of the IAC C_t values for the sample replicates are more than 4.0 C_ts greater than the negative control IAC C_t value AND the corresponding sample is positive for norovirus and/or hepatitis A virus, the Mengovirus RTqPCR does not have to be repeated. If norovirus or hepatitis A virus is detected in a sample that has inhibition present in the RTqPCR reaction and has log amplification, this sample does not need to be repeated for norovirus or hepatitis A virus RTqPCR and would be considered positive. Repeating RT-qPCR reactions due to inhibition is to ensure that you do not have false negatives.

Tables

Table B5.1. Primer and Probe Sequences for Mengovirus and Internal Amplification Control RNA

Identification	Primers	Location∞
MengoF[a, β]	5’ GCG GGT CCT GCC GAA AGT 3’	110-127
MengoR[a,β]	5’ GAA GTA ACA TAT AGA CAG ACG CAC AC 3’	239-264
IC46F[b,c]	5’GAC ATC GAT ATG GGT GCC G-3’	N/A
IC194R[b,c]	5’-AAT ATT CGC GAG ACG ATG CAG-3’	N/A
	Probes	
MengoP[a, β]	Cy5- 5’ ATC ACA TTA CTG GCC GAA GC 3’ -IB-RQ*	203-221
IACP[b,c]	TxR –TCT CAT GCG TCT CCC TGG TGA ATG TG -IB RQ*	N/A

[a] Pinto et. al 2009 et al.
[b]Internal Amplification Control (IAC) primers and probes are covered by U.S. Patent Application 0060166232
[c]Depaola, Jones, Woods, et al. 2010.

[β]Based on GenBank accession # DQ2946335

*IB RQ- Iowa Black RQ

Table B5.2. ABI 7500 Amplification Reaction Components and Master Mix Volume for Mengovirus

Reagent	**Initial Concentration**	**Volume per 25 µl reaction**	**Final Concentration**
RNase Free H_20		9.425 µl	-
5X OneStep RT-PCR Buffer	5X	5.0 µl	1X
$MgCl_2$	50 mM	0.75 µl	1.5 mM
dNTP Mix	10 mM	1 µl	0.4 mM
MengoF	10 µM	0.50 µl	0.2 µM
MengoR	10 µM	0.50 µl	0.2 µM
IC 46F	10 µM	0.1875 µl	0.075 µM
IC 194R	10 µM	0.1875 µl	0.075 µM
Mengo Probe	10 µM	0.625 µl	0.25 µM
IACP	10 µM	0.375 µl	0.15 µM
OneStep RT- qPCR Enzyme Mix		1.00 µl	
Superasin	20 Units/µl	0.25 µl	5 Units
FAM ref dye	500 nM	2 µl	0.04 µM
Internal Amplification Control RNA		*0.2 µl	-
RNA/Extract		3µl	

*Amount varies with concentration of IAC RNA. The amount of IAC template needs to be adjusted based on the prepared stock concentration to report Cycle threshold (C_t) of 20-25 when no inhibition is present in the reaction. The required concentration was provided to each laboratory participating in the validation study.

˜With the addition of 1.5 mM $MgCl_2$ the final concentration per reaction is 4.0 mM $MgCl_2$

Section B6: Buffer and Recipes

1. **Working Concentration FAM Dye**

FAM dye (BioRAD)	0.5 µl
Primer TE	1000 µl

Add mixture to 1.5 ml DNase/RNase free dark tube, mix well and make 250 µl aliquots in DNase/RNase dark tubes. Store at 4 °C for 3 months.

2. **Primer TE (10 mM Tris, 0.1 mM EDTA, pH 8.0)**

1 M Tris pH 8.0	100 ul
0.05 M EDTA	20 ul
PCR-grade water (Dnase/Rnase free)	9.88 ml

Prepare in sterile 50 ml conical tube. Store at room temperature.

3. **1X TAE buffer**

50X TAE	100 ml
DI Water	4900 ml

Combine and mix well before use.

Section B7: Positive Controls Preparation

Sample positive control preparation from propagated or commercially acquired virus (murine norovirus, mengovirus, hepatitis A virus).

1. For murine norovirus and mengovirus, obtain 1 aliquot of the diluted extraction control as prepared in section A5. For hepatitis A virus, prepare as indicated for the extraction controls.
2. Follow manufactures instruction for QIAamp Viral RNA Mini Kit or RNesay kit for extraction of RNA.
3. For norovirus GI and GII transcripts, prepare according to manufacturer's instructions.
4. Add Superasin to extraction RNA for a final concentration of 1%. Aliquot for use as your PCR positive control working stock. Store positive control at ≤ -20 °C for short term (≤ 30 days) and -70 °C for long term.

***Note: Recommended C_t for all positive controls is ≤ 35.**

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