Concentration, Extraction, and Detection of Norovirus and Hepatitis A virus in Scallops and Finfish Meat

In the US, hepatitis A (HAV) outbreaks associated with the consumption of molluscan shellfish have not occurred in over 10 years. In addition, there have not been any reports in the US of HAV infection where scallops were the implicated vehicle. However, in June of 2016, a cluster of HAV illnesses associated with the consumption of imported uncooked bay scallops occurred. The method described here was developed by CFSAN's Gulf Coast Seafood Laboratory as a matrix extension to BAM 26B. This protocol will provide concentration and extraction of enteric viruses from scallop and finfish meat. The concentration and extraction uses ultracentrifugation and Qiagen's® QIAamp Viral RNA Mini Kit, respectively. This method should be used in conjunction with FDA BAM Chapter 26B RT-qPCR assays for the detection of HAV and MNV. In this document, instructions are also provided for the validated RT-qPCR assays for the detection of NoV GI and GII using the Cepheid SmartCycler® System and the detection of NoV GI, NoV GII, and HAV on the ABI 7500. Valid sample results are contingent upon the successful detection of the MNV extraction control from the sample being tested.

1. Materials, Reagents, Equipment, and Supplies

1. Glycine (Sigma G-7126 or equivalent; TLC grade or better)
2. Nuclease free-water (Applied Biosystems AM9937 or equivalent)
3. NaCl (Sigma S3014 or equivalent)
4. KCl (Sigma P9541 or equivalent)
5. KH_2PO_4 (Sigma P9791 or equivalent)
6. Na_2HPO_4 (Sigma S5011 or equivalent)
7. Chloroform (Sigma C2432 or equivalent)
8. Tris base (Fisher Scientific BP152-1 or equivalent)
9. Ethanol (95-100%)
10. Biological Safety Cabinets (BSC- 2 Type A2 or higher air exchange rate)
11. -70˚C or -80 ˚C Ultra low freezer
12. RNase-free latex or nitrile gloves
13. Vortex Mixer
14. Pop micro-centrifuge
15. PBS (tissue culture grade) formula provided or PBS tissue culture grade (Sigma P5493), 10X solution to be diluted to 1X
16. Calibrated pipettors dedicated for RNA virus extraction (Rainin PR10, 17008649; PR20, 17008650; PR100, 17008651; PR1000, 17008653 or equivalent set)

17. Qiagen QIAamp Viral RNA Mini Kit (Cat no. 52904)
18. Qiagen QIAshredder (Cat no.79654)
19. Qiagen collection tubes (Cat no. 19201)
20. Filter-barrier aerosol resistant pipette tips (USA Scientific 1120-3810, 1120-8810, 1126-7810 or equivalent)
21. Bench top cooling block, pre-chilled to 4°C (preferable) or ice bucket with ice
22. Micro-centrifuge (Eppendorf 5145 D or equivalent)
23. Micro-centrifuge tubes, 1.5-1.7 ml, siliconized, certified DNase & RNase free (Life Technologies AM12450 or equivalent)
24. Micro-centrifuge tubes, 2.0 ml, siliconized, certified DNase & RNase free (Life Technologies AM12475 or equivalent)
25. High speed centrifuge (ThermoFisher Scientific 46910)
26. FY14 50 carbon fiber rotor (ThermoFisher Scientific 46922 or equivalent)
27. 50ml conical tubes (Fisher Scientific 14-959-49A or equivalent)
28. Ultra-centrifuge (Sorval WX90 or equivalent)
29. Carbon fiber rotor F40L, 8 x 100 ml (Thermofisher 096-087057 or equivalent)
30. 70 mL polycarbonate ultracentrifuge tubes w/aluminum cap tubes for F40L rotor (Fisher Scientific 010-1333 or equivalent)
31. Shaker, orbital or side-to-side (Labsource S16 304 or equivalent)
32. Balance (sensitivity of 0.01 g)
33. One Step PCR Inhibitor Removal Kit (Zymo Research D6030)
34. Whirl Pak filter bags 6x9 inch (Fisher Scientific 01-812-69 or equivalent)
35. 95% Ethanol (Sigma E7023)
36. -70˚C or -80 ˚C Ultra low freezer
37. RNase-free latex or nitrile gloves
38. Vortex mixer
39. Smart Cycler tube holder
40. Smart Cycler cold block for Smart cycler tubes (-20 ºC)
41. Adjustable Micropipettors (0.2 – 1000 µl), dedicated for RNA work only
42. Filter barrier aerosol resistant micropipettor tips DNase/RNase free (0.2 – 1000 µl)
43. Vortex Mixer
44. Smart Cycler Reaction Tubes, 25-µl capacity (Fisher Scientific 11-400-3)
45. Latex or nitrile gloves- Powder Free
46. Cepheid Smart Cycler II
47. Qiagen QIAamp Viral RNA Mini Kit (Cat no. 52904)
48. Qiagen QIAshredder (Cat no.79654)
49. Qiagen collection tubes (Cat no. 19201)
50. OneStep™ PCR Inhibitor Removal Kit (Zymo Research D6030)
51. ABI 7500 or 7500 FAST with software v2

52. Smart Cycler tube mini-centrifuge with Smart Cycler adapter
53. ABI 96 well plates (Life Technologies) cat # 4346906
54. ABI plates cover (Life Technologies) cat #4311971
55. Mini Plate Spinner (Fisher Scientific)
56. Pop micro-centrifuge
57. 96 well cool rack (Sigma Aldrich #Z606634-1EA) or equivalent
58. 50ml Falcon tubes (Fisher 14-959-49A)
59. 2.0ml microcentrifuge tubes DNase/RNase free (USA Scientific 1620-2799)
60. 70ml ultracentrifuge tubes (Fisher NC 9959232)
61. Ultracentrifuge with applicable speeds of ≥170,100 x g and with rotors capable of holding 70ml polypropylene ultracentrifuge tubes.
62. Microcentrifuge and DNase/RNAse free microcentrifuge tubes (Applied Biosystems AM12350)1.5 ml (AM12450) 2.0ml (12475)
63. Disposable transfer pipettes (Fisher 13-711-22)
64. Primer TE
65. Qiagen OneStep RT-PCR Kit; Cat No. 210210 (25 reactions) or 210212 (100 reactions)
66. Ambion Superase·In (20 units/µl); Life Technologies Cat No. AM2694 (2,500 U) or AM2696 (10,000 U) Life Technologies
67. Orbital shaker (Fisher Scientific 11-676-231 or equivalent)
68. 50 mM $MgCl_2$ or 25 mM $MgCl_2$
69. Internal Control RNA (available from BioGX Cat No. 750-0001)
70. positive controls (Quantitative Synthetic Norovirus GI— ATCC® VR-3234SD, Quantitative Synthetic Norovirus GII—ATCC® VR-3235SD, HAV RNA—ATCC VR-1402 and murine norovirus RNA—ATCC PTA-5935)
71. negative RT-qPCR control (Nuclease free-water Applied Biosystems AM9937)
72. Fluorescein calibration dye (BioRad cat#1708780)
73. Extraction control murine norovirus (ATCC PTA-5935)

2. Concentration and Extraction Procedure

Note: *for large uncut finfish, cut from the surface to obtain 50 grams.

1. Add 50 g ± 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 to sample (preparation details described in BAM 26B).

3. Add 50 ml of 0.05 M Glycine/Tris Buffer (Appendix A) and tightly close.

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

5. Decant 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 ºC.

7. Decant supernatant into a 70 ml Ultra-centrifuge tube.

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

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

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

11. Label QIAshredder and QIAamp Spin column with sample number.

12. Carefully pipette and discard supernatant (**should see pellet on side of tube or pellet may dislodge and fall to bottom).**

Note: *There may be a clear gelatinous substance; this will be resuspended with pellet.

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

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

15. Evenly distribute aqueous layer into two separate 2.0 ml DNase/RNase free microcentrifuge tubes. **Use one of the tubes for RNA extraction. Store second tube at -70° C.**

3. RNA Extraction

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

1. Add 560 µl prepared Buffer AVL with carrier RNA to tube from section 2, step 15.

   (see instructions in Appendix A on how to prepare the AVL with carrier RNA)

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 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 ethanol (50%) 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. Add 500 µl Buffer AW1. Incubate for 10 min. Centrifuge 1 min at 8000 x *g*. Discard flow through and collection tube.

13. Transfer the QIAamp mini column into a new 2 ml collection tube. Add 500 µl Buffer AW2 onto the QIAamp Mini column. Centrifuge at full speed (~20,000 x *g*) for 3 min. Discard flow through and collection tube.

14. Transfer the QIAamp mini column into a new 2 ml collection tube. Centrifuge at full speed (~20,000 x *g*) for 1 min to dry column.

15. 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 8000 x g.

16. Pipet 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*.

17. Place tube with RNA (step 16) on ice to prepare Zymo column.

18. Prepare Zymo column per manufactures instructions.

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

20. Transfer RNA from step 17 to prepared Zymo One Step RT-PCR inhibitor remover column.

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

22. Proceed with RT-qPCR as described in BAM 26B or freeze at -80°C for long term storage.

4. Buffer and Reagent Recipes

1. 0.05 M Glycine/0.05 M Tris (pH 9.5)

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

QS to 1L with distilled water
Adjust pH to 9.5, sterilize 121°C for 15 min and store at 4 °C

2. PBS (tissue culture grade; t.c.)

10X PBS	100 ml
Sterile distilled water	900 ml

Store at 4°C

3. PBS (tissue culture grade; t.c.)

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

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

4. 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.

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

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

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

6. Carrier RNA

Buffer AVE	310 µl
310 µg carrier RNA	310 µg

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

7. 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).

***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

RT-qPCR Assays Smart Cyclers

1. **Outlined MNV RT-qPCR Assay for Smart Cycler (refer to FDA BAM 26B for primers, probes, master mix, and extraction set-up)**

   A. Sample Preparation

   NOTE: Sample preparation should be conducted in an area separate from the PCR prep area. Assembly of master mix should be done in a Master Mix PCR hood or BSC hood that has been decontaminated with RNase Away™, Nucleoclean™, RNaseZap™, or similar product and UV irradiated for 20 minutes. Change gloves often and when exiting and/or reentering the hood. Always use aerosol resistant pipette tips for PCR.

   B. Murine norovirus Protocol

   Reverse transcription: 50°C for 3000 sec

   Activation: 95°C for 900 sec

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

   C. Reaction Set-Up

   NOTE: Always use aerosol resistant pipette tips for PCR.

   a. Label all necessary Smart Cycler reaction tubes and place in Smart Cycler cold block.

   b. Thaw primer solutions, probe solutions, and buffer mix and place them in bench top cool block in reagent hood.

   c. Vortex for 5-10 sec at setting 7-10, then pop spin centrifuge 3-5 seconds to settle the liquid to the bottom of the tube. Place in ice or bench top cooler. Keep Enzyme Mix in cooling block at all times, these enzymes do not need to be defrosted.

   d. Prepare Master Mix for all sample and control reactions as in MNV MM sheet. Keep all thawed components, reagents, controls and master mixes **in cooling block**.

e. Add 3µl of negative control (PCR water) to the appropriate reaction tube

NOTE: Viral RNA templates should be added to reaction tubes in a designated area separate from location where master mixes are prepared.

a. Proceed to template hood and thaw IAC RNA and Sample RNA in Template Hood. Briefly spin the tubes 3-5 seconds in a POP micro-centrifuge to settle the liquid at the bottom of the tube. Add appropriate volume of IAC, (0.2 ul/rxn) to NoV Master Mix (keep cold); Vortex briefly & Pulse spin.

b. Add 22 µl Master Mix to each pre-labeled Smart Cycler Reaction Tube.

c. Add 3µl of sample template to the appropriate reaction tubes

d. Add 3µl of positive control template to the appropriate reaction tube

e. Close reaction tube, briefly spin to mix bring down reagents.

f. Place reactions tubes in the Smart Cycler and create run. Make sure the appropriate dye set (FCTC25) and protocols (see creating protocol) are selected for each site. Name the run with the assay, sample number, analysts initials.

g. Start run; the entire reaction time for this assay is approx 3 h.

D. Data Analysis

h. For results analysis, default instrument settings will be used, except the threshold is set at 10 for all channels utilized.

i. On the SmartCycler II Instrument, set the following Analysis Settings for TxRed and Cy5 channels. Update analysis settings if they are changed before recording results.

j. 1.Usage: Assay

k. 2.Curve Analysis: Primary

l. 3. Threshold Setting: Manual

m. 4. Manual Threshold Fluorescence Units: 10.0

n. 5. Auto Min Cycle: 5

o. 6. Auto Max Cycle: 10

p. 7. Valid Min Cycle: 3

q. 8. Valid Max. Cycle: 60

r. 9. Background subtraction: ON

s. 10. Boxcar Avg. Cycles: 0

t. 11. Background Min. Cycle: 5

u. 12. Background Max. Cycle: 40

v. 13. Max Cycles 45

w. Any sample which crosses the threshold in the Cy5 (Ch. 4) channel will demonstrate detection of MNV.

x. The IAC will report in Channel 3 (TxRed).

Data Interpretation Murine Norovirus Detection Assay

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

1. Sample is "invalid" and must be repeated if:
   a. The negative RT-qPCR control sample demonstrates positive results crossing the Cy5 threshold or if the IAC is negative
   b. The RT-qPCR positive control is negative for MNV
   c. The MNV RT-qPCR is negative in spiked samples
   d. The average of the IAC Ct values for the sample replicates are more than 4.0 Ct's greater than the Negative Control IAC Ct value, the RT-qPCR assay must be repeated using remaining RNA or RNA from a newly extracted tube with a 1µl template in the RT-qPCR reaction in triplicate. If the 1µl template reactions yields an average IAC Ct values greater than 4.0 Cts higher than the Negative Control IAC Ct value, the sample must be repeated from the beginning using additional food sample. With the new sample, the concentrates will be split into 5 tubes (refer to Work Instructions) and complete RT-qPCR with 1µl 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 spikes matrices
   d. Internal amplification control (IAC) is positive in all reactions and average of the IAC Ct values for sample is within 4.0 Ct's of the Negative Control IAC Ct Value.

2. Outlined HAV RT-qPCR Assay for Smart Cycler (refer to FDA BAM 26B for primers, probes, master mix, and extraction set-up)

A. Sample Preparation

NOTE: Sample preparation should be conducted in an area separate from the PCR prep area. Assembly of master mix should be done in a Master Mix PCR hood or BSC hood that has been decontaminated with RNase Away™, Nucleoclean™, RNaseZap™, or similar product and UV irradiated for 20 minutes. Change gloves often and when exiting and/or reentering the hood. Always use aerosol resistant pipette tips for PCR.

B. Hepatitis A Virus Protocol Smart Cycler

Reverse transcription: 50°C for 3000 sec

Activation: 95°C for 900sec

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

C. Reaction Set-Up

NOTE: Always use aerosol resistant pipette tips for PCR.

a. Label all necessary Smart Cycler reaction tubes and place in Smart Cycler cold block.

b. Thaw primer solutions, probe solutions, and buffer mix and place them in bench top cool block in reagent hood.

c. Vortex for 5-10 sec at setting 7-10, then pop spin centrifuge 3-5 seconds to settle the liquid to the bottom of the tube. Place in ice or bench top cooler. Keep Enzyme Mix in cooling block at all times, these enzymes do not need to be defrosted.

d. Prepare Master Mix for all sample and control reactions as in HAV MM sheet. Keep all thawed components, reagents, controls and master mixes **in cooling block**.

e. Add 3µl of negative control (PCR water) to the appropriate reaction tube

f. *NOTE: Viral RNA templates should be added to reaction tubes in a designated area separate from location where master mixes are prepared.*

g. Proceed to template hood and thaw IAC RNA and Sample RNA in Template Hood. Briefly spin the tubes 3-5 seconds in a POP micro-centrifuge to settle the liquid at the bottom of the tube. Add appropriate volume of IAC, (0.2 ul/ rxn) to NoV Master Mix (keep cold); Vortex briefly & Pulse spin.

h. Add 22 µl Master Mix to each pre-labeled Smart Cycler Reaction Tube.

i. Add 3µl of sample template to the appropriate reaction tubes

j. Add 3µl of positive control template to the appropriate reaction tube

k. Close reaction tube, briefly spin to mix bring down reagents.

l. Place reactions tubes in the Smart Cycler and create run. Make sure the appropriate dye set (FCTC25) and protocols (see creating protocol) are selected for each site. Name the run with the assay, sample

m. number, analysts initials.

n. Start run; the entire reaction time for this assay is approx. 3 h.

D. Data Analysis

o. For results analysis, default instrument settings will be used, except the threshold is set at 10 for all channels utilized.

p. On the SmartCycler II Instrument, set the following Analysis Settings for TxRed and Cy5 channels. Update analysis settings if they are changed before recording results.

q. 1.Usage: Assay

r. 2.Curve Analysis: Primary

s. 3. Threshold Setting: Manual

t. 4. Manual Threshold Fluorescence Units: 10.0

u. 5. Auto Min Cycle: 5

v. 6. Auto Max Cycle: 10

w. 7. Valid Min Cycle: 3

x. 8. Valid Max. Cycle: 60

y. 9. Background subtraction: ON

z. 10. Boxcar Avg. Cycles: 0

aa. 11. Background Min. Cycle: 5

bb. 12. Background Max. Cycle: 40

cc. 13. Max Cycles 50

dd. Any sample which crosses the threshold in the Cy5 (Ch. 4) channel will be considered positive for HAV.

ee. The IAC will report in Channel 3 (TxRed).

Data Interpretation HAV Detection 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 HAV

3. Samples are invalid if:
   a. If the negative RT-qPCR control sample demonstrates positive results crossing the Cy5 threshold or if the IAC is negative, the RT-qPCR assay must be repeated.
   b. The RT-qPCR positive control is negative for HAV
      c. The average of the IAC Ct values for the sample replicates are more than 4.0 Ct's greater than the Negative Control IAC Ct value, the RT-qPCR assay must be repeated using remaining RNA or RNA from a newly extracted saved tube with a 1µl RT-qPCR reaction in triplicate. If the repeat of the newly extracted sample

yields average IAC Ct values 4.0 Cts higher than the Negative Control IAC Ct value, the sample must be repeated from the beginning using additional food sample. With the new repeat, the tubes will be split into 5 tubes and complete RT-qPCR with 1ul reactions in triplicate.

3. Outlined NoV RT-qPCR Assay for Smart Cycler

Norovirus Primers and Probes

All NoV probes and primers were commercially synthesized (Integrated DNA Technologies, Coralville, IA). The NoV GI probe is labeled 5' with Cy5 reporter dye and 3' with Iowa Black RQ as a quencher. The NoV GII probe is labeled 5' with Cy3 reporter dye and 3' with Iowa Black RQ as a quencher. The IAC probe is labeled 5' with TxRed reporter dye and 3' with Iowa Black RQ as a quencher. All primers and probes are hydrated in sterile primer TE buffer (see Appendix E) to 100 µM concentration. Ten µM working stocks are prepared from the 100 µM stock solution and are stored at -20 °C in a frost free freezer.

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

Identification	**Primers**	**Location**[∞,β]
COG1F[a,∞]	5' CGY TGG ATG CGN TTY CAT GA 3'	5287-5306
COG1R[a,∞]	5' CTT AGA CGC CAT CAT CAT TYA C 3'	5350-5371
COG2F[a,β]	5' CAR GAR BCN ATG TTY AGR TGG ATG AG 3'	5003-5028
COG2R[a,β]	5' TCG ACG CCA TCT TCA TTC ACA 3'	5080-5100
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' AGA TYG CGA TCY CCT GTC CA 3' -IB-RQ*	5317-5336
COGP1b[a,∞]	Cy5- 5' 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 2. Amplification Reaction Components and Master Mix Volume for NoV

Reagent	Initial Concentration	Volume per 25 µl reaction	Final Concentration
RNase Free H_20		9.3 µl	-
5 X OneStep RT-PCR Buffer	5X	5.0 µl	1 X
$MgCl_2$~	50mM	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	0.15µM
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		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 a Cycle threshold (C_t) of 20-25 PCR cycles when no inhibition is present in the reaction.
~With the addition of 1.5 mM MgCl, the final concentration per reaction is 4.0mM $MgCl_2$

A. Sample Preparation

NOTE: Sample preparation should be conducted in an area separate from the PCR prep area. Assembly of master mix should be done in a Master Mix PCR hood or BSC hood that has been decontaminated with RNase Away™, Nucleoclean™, RNaseZap™, or similar product and UV irradiated for 20 minutes. Change gloves often and when exiting and/or reentering the hood. Always use aerosol resistant pipette tips for PCR.

B. Norovirus Protocol Smart Cycler

Reverse transcription: 50°C for 3000 sec

Activation: 95°C for 900sec

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

C. Reaction Set-Up

NOTE: Always use aerosol resistant pipette tips for PCR.

a. Label all necessary Smart Cycler reaction tubes and place in Smart Cycler cold block.

b. Thaw primer solutions, probe solutions, and buffer mix and place them in bench top cool block in reagent hood.

c. Vortex for 5-10 sec at setting 7-10, then pop spin centrifuge 3-5 seconds to settle the liquid to the bottom of the tube. Place in ice or bench top cooler. Keep Enzyme Mix in cooling block at all times, these enzymes do not need to be defrosted.

d. Prepare Master Mix for all sample and control reactions as in Noro MM sheet. Keep all thawed components, reagents, controls and master mixes **in cooling block**.

f. Add 3µl of negative control (PCR water) to the appropriate reaction tube

g. *NOTE: Viral RNA templates should be added to reaction tubes in a designated area separate from location where master mixes are prepared.*

h. Proceed to template hood and thaw IAC RNA and Sample RNA in Template Hood. Briefly spin the tubes 3-5 seconds in a POP micro-centrifuge to settle the liquid at the bottom of the tube. Add appropriate volume of IAC, (0.2 ul/rxn) to NoV Master Mix (keep cold); Vortex briefly & Pulse spin.

i. Add 22 µl Master Mix to each pre-labeled Smart Cycler Reaction Tube.

j. Add 3µl of sample template to the appropriate reaction tubes

k. Add 3µl of positive control template to the appropriate reaction tube

l. Close reaction tube, briefly spin to mix bring down reagents.

m. Place reactions tubes in the Smart Cycler and create run. Make sure the appropriate dye set (FCTC25) and protocols (see creating protocol) are selected for each site. Name the run with the assay, sample number, analysts initials.

n. Start run; the entire reaction time for this assay is approx 3 h.

D. Data Analysis

o. For results analysis, default instrument settings will be used, except the threshold is set at 10 for all channels utilized.

p. On the SmartCycler II Instrument, set the following Analysis Settings for TxRed, Cy3, and Cy5 channels. Update analysis settings if they are changed before recording results.

q. 1.Usage: Assay

r. 2.Curve Analysis: Primary

s. 3. Threshold Setting: Manual

t. 4. Manual Threshold Fluorescence Units: 10.0

u. 5. Auto Min Cycle: 5

v. 6. Auto Max Cycle: 10

w. 7. Valid Min Cycle: 3

x. 8. Valid Max. Cycle: 60

y. 9. Background subtraction: ON

z. 10. Boxcar Avg. Cycles: 0

aa. 11. Background Min. Cycle: 5

bb. 12. Background Max. Cycle: 40

cc. 13. Max Cycles 50

dd. Any sample which crosses the threshold in the Cy3 (Ch. 2) channel will be considered positive for NoV genogroup II. Any sample which crosses the threshold in the Cy5 (Ch. 4) channel will be considered positive for NoV genogroup I.

ee. The IAC will report in Channel 3 (TxRed).

Data Interpretation for Detection NoV

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.

1. 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.

2. Sample is "positive" if:
   a. RT-qPCR negative control is negative for GI and GII,
   b. RT-qPCR control is positive for GI and GII,
   c. Unknown sample is positive for GI and/or GII

3. 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, the RT-qPCR assay must be repeated.
   b. The RT-qPCR positive control is negative for GI and/or GII
      c. The average of the IAC Ct values for the sample replicates are more than 4.0 Ct's greater than the Negative Control IAC Ct value, the RT-qPCR assay must be repeated using remaining RNA or RNA from a newly extracted saved tube with a 1µl RT-qPCR reaction in triplicate. If the repeat of the newly extracted sample yields average IAC Ct values 4.0 Cts higher than the Negative Control IAC Ct value, the sample must be repeated from the beginning using additional food sample. with the new repeat, the tubes will be split into 5 tubes and complete RT-qPCR with 1ul reactions in triplicate.

4. RT-qPCR Assays ABI 7500

Outlined MNV RT-qPCR Assay for ABI 7500 (refer to FDA BAM 26B for primers and probes. Refer to the proceeding Table 3 for master mix components)

A. Cycling Conditions

Reverse transcription: 50°C for 3000 sec Activation: 95°C for 900sec
Cycling: 45 cycles of 95°C for 15 sec, 55°C for 20 sec, 62°C for 60 sec with optics on

B. Reaction Set-Up

a. Thaw primer solutions, probe solutions, and buffer mix and place them in bench top cool block in reagent hood.

b. Vortex for 5-10 sec at setting 7-10, then spin centrifuge 3-5 seconds in a personal micro-centrifuge to settle the liquid to the bottom of the tube. Place in ice or bench top cooler.

c. Prepare Master Mix in a designated clean area for all sample and control reactions. *Keep all thawed components, reagents, controls and master mixes in cooling block.

d. Proceed to template area and thaw IAC RNA and sample RNA in Template Hood/Area.

e. Briefly spin the tubes 3-5 seconds in a personal micro-centrifuge to settle the liquid at the bottom of the tube.

f. Add appropriate volume of IAC, (0.2 ul/rxn) to Master Mix (keepcold); Vortex briefly & pulse spin.

g. Add 22 µl Master Mix to each sample well.

h. Add 3µl of negative control (PCR water) to the designated well. *Note: in an effort to prevent cross contamination, it is recommended that after adding the negative control, youcover the plate with ABI plate cover and lift the cover as you add the template*

i. Add 3µl of sample template to the three designated wells

j. Add 3µl of positive control template to the designated well.

k. Cover plate with sealing film or strip caps and then briefly spin in 96 well plate spinner to bring down reagents.

C. Instrument Set-Up

a. Place 96 well plate in ABI instrument and create run. Name the run with the assay, sample number, analyst initials.

b. Open ABI Software and Click on Icon for Advanced Setup

c. Fill out the "Experiment Name" field (other fields are optional)

d. Select 7500 (FAST) or 7500 depending on which machine you are using

e. Select "Quantitation Standard Curve"

f. Select "TaqMan Reagents"

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

h. Click "Plate Set Up"

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

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

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

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

Note this is not the amount of reactions, but rather the total samples you have. For example, if you have 2 samples in triplicate you have 2 samples under this area NOT 6

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

n. Click "Assign Targets & Samples" tab

o. In the plate layout area highlight the locations where you have samples, while area is highlighted check both targets located in the target area to the left of the plate layout.

p. Highlight each plate area with a single sample and check the sample located under the "assign samples to the selected well(s)" area located to the left of the plate layout until all samples are labeled

q. Under "Select the dye to use as the passive reference" scroll to FAM

r. Select "Run Method"

s. Change the reaction volume per well to 25 µl

t. Click tabular view

u. You will need 2 holding stages and 1 cycling stage with 3 steps (add or remove stages and steps as needed)

v. In the first holding stage set the parameters at 3000 seconds (50 minutes) at 50°C

w. In the second holding stage set the parameters at 900 seconds (15 minutes) at 95°C

x. In the cycling stage set the repeats to 45 cycles

y. 1st stage set at 15 seconds at 95°C

z. 2nd stage set at 20 seconds at 55°C (make sure you unclick the icon for collecting data)

aa. 3rd stage set at 60 seconds (1 minute) at 62°C (click the icon for collecting data at "collect data on hold")

bb. Save the run method – Save as MNV Multiplex (after you have saved the method you will no longer have to set it up, you may now simply select "Open Run Method" and select the saved method. However, you will have to click "Yes" on the pop up box)

cc. Click the "Analysis" tab on the right

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

ee. Click on each target so that it is highlighted

ff. For Cy5 (MNV) – uncheck the "Use Default Settings" to the right under "CT Settings for Target 1"

gg. Uncheck "Automatic Threshold" – set Threshold to 0.1

hh. Uncheck "Automatic Baseline" – set "Baseline Start Cycle" to 3 and "End Cycle" to 10

ii. For TxR (Internal Control) – Repeat as for Cy5

jj. Click "Apply Analysis Settings"

kk. Click "Run" tab on the right

ll. Click green "Start Run" box

mm. Pop up screen will prompt you to save the data. Save the data to your designated files.

Data Interpretation

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

1. Sample is "invalid" and must be repeated 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 MNV
   c. The MNV RT-qPCR is negative in any sample
   d. The average of the IAC Ct values for the sample replicates are more than 4.0 Ct's greater than the Negative Control IAC Ct value, the RT- qPCR assay must be repeated using remaining RNA or RNA from a newly extracted tube with a 1µl template in the RT-qPCR reaction in triplicate. If the 1µl template reactions yields an average IAC Ct values greater than 4.0 Ct higher than the Negative Control IAC Ct value, the sample must be repeated from the beginning using additional food sample. With the new sample, the concentrates will be split into 5 tubes (refer to Work Instructions) and complete RT-qPCR with 1µl 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 spikes matrices
   d. Internal amplification control (IAC) is positive in all reactions and average of the IAC Ct values for sample is within 4.0 Ct's of the Negative Control IAC Ct Value.

Table 3. ABI 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	-
5 X OneStep RT-PCR Buffer	5X	5.0 µl	1 X
$MgCl_2$~	50mM	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	0.15µM
OneStep RT-PCR Enzyme Mix		1.00 µl	
Superasin	20 Units/µl	0.25 µl	5 Units
FAM ref dye	500nM	2 µl	0.04 µM
Internal Amplification Control RNA		*0.2 µl	-
RNA		3µl	

∞Sequences can be found in FDA BAM Chapter 26B

*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 PCR cycles 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 4.0mM MgCl2

5. Outlined HAV RT-qPCR Protocol ABI 7500 (primers and probes listed in FDA BAM 26B, master mix components listed in Table 4).

A. Cycling Conditions

Reverse transcription: 50°C for 3000 sec Activation: 95°C for 900sec
Cycling: 50 cycles of 95°C for 10sec, 53°C for 25sec, 64°C for 70sec with optics on

B. Reaction Set-Up

a. Thaw primer solutions, probe solutions, and buffer mix and place them in bench top cool block in reagent hood.

b. Vortex for 5-10 sec at setting 7-10, then spin centrifuge 3-5 seconds in a personal micro-centrifuge to settle the liquid to the bottom of the tube. Place in ice or bench top cooler.

c. Prepare Master Mix in designated clean area for all sample and control reactions. *Keep all thawed components, reagents, controls and master mixes in cooling block.

d. Proceed to template area and thaw IAC RNA and sample RNA in Template Hood/Area.

e. Briefly spin the tubes 3-5 seconds in a personal micro- centrifuge to settle the liquid at the bottom of the tube.

f. Add appropriate volume of IAC, (0.2 ul/ rxn) to Master Mix (keep cold); Vortex briefly & pulse spin.

g. Add 22 µl Master Mix to each sample well.

h. Add 3µl of negative control (PCR water) to the designated well.

Note: in an effort to prevent cross contamination, it is recommended that after adding the negative control, you cover the plate with ABI plate cover and lift the cover as you add the template

i. Add 3µl of sample template to the three designated wells

j. Add 3µl of positive control template to the designated well.

k. Cover plate with sealing film or strip caps and then briefly spin in 96 well plate spinner to bring reagents down to the bottom.

C. Instrument Set-Up

a. Place 96 well plate in ABI instrument and create run. Name the run with the assay, sample number, analyst initials.

b. Open ABI Software and Click on Icon for Advanced Setup

c. Fill out the "Experiment Name" field (other fields are optional)

d. Select 7500 (FAST) or 7500 depending on which machine you are using

e. Select "Quantitation Standard Curve"

f. Select "TaqMan Reagents"

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

h. Click "Plate Set Up"

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

j. Fill Target 1 with HAV, Target 2 with IAC

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

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

Note this is not the amount of reactions, but rather the total samples you have. For example, if you have 2 samples in triplicate you have 2 samples under this area NOT 6

m. Fill in your samples names in each of the areas provided

n. Click "Assign Targets & Samples" tab

o. In the plate layout area highlight the locations where you have samples, while area is highlighted check all three targets located in the target area to the left of the plate layout.

p. Highlight each plate area with a single sample and check the sample located under the "assign samples to the selected well(s)" area located to the left of the plate layout until all samples are labeled

q. Under "Select the dye to use as the passive reference" scroll to FAM

r. Select "Run Method"

s. Change the reaction volume to 25 µl per well

t. Click tabular view

u. You will need 2 holding stages and 1 cycling stage with 3 steps (add or remove stages and steps as needed)

v. In the first holding stage set the parameters at 3000 seconds (50 minutes) at 50°C

w. In the second holding stage set the parameters at 900 seconds (15 minutes) at 95°C

x. In the cycling stage set the repeats to 50 cycles

y. 1st stage set at 10 seconds at 95°C

z. 2nd stage set at 25 seconds at 53°C (make sure you unclick the icon for collecting data)

aa. 3rd stage set at 70 seconds (1 minute; 10 seconds) at 64°C (click the icon for collecting data at "collect data on hold")

bb. Save the run method – Save as HAV Multiplex (after you have saved the method you will no longer have to set it up, you may now simply select "Open Run Method" and select the saved method. However, you will have to click "Yes" on the pop up box)

cc. Click the "Analysis" tab on the right

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

ee. Click on each target so that it is highlighted

ff. For Cy5 (HAV) – uncheck the "Use Default Settings" to the right under "CT Settings for Target 1"

gg. Uncheck "Automatic Threshold" – set Threshold to 0.1

hh. Uncheck "Automatic Baseline" – set "Baseline Start Cycle" to 3 and "End Cycle" to 10

ii. For TxR (Internal Control) – Repeat as for Cy5

jj. Click "Apply Analysis Settings"

kk. Click "Run" tab on the right

ll. Click green "Start Run" box

mm. Pop up screen will prompt you to save the data. Save the data to your designated files.

Data Interpretation

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

Table 4. ABI 7500 Amplification Reaction Components for HAV∞

Reagent	Initial Concentration	Volume per 25 µl reaction	Final Concentration
RNase Free H_20		9.05µl	-
5 X OneStep RT-PCR Buffer	5X	5.0 µl	1 X
$MgCl_2$~	50mM	0.75 µl	1.5 mM
dNTP Mix	10 mM	1 µl	0.4 mM
HAV2F	10 µM	0.75 µl	0.3 µM
HAV1R	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	0.15µM
OneStep RT-PCR Enzyme Mix		1.00 µl	
Superasin	20 Units/µl	0.25 µl	5 Units
FAM ref dye	500nM	2 µl	0.04µM
Internal Amplification Control RNA		*0.2 µl	-
RNA		3µl	

∞Sequences can be found in FDA BAM Chapter 26B
*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 PCR cycles 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 4.0mM $MgCl_2$

6. Outlined NoV RT-qPCR Protocol ABI 7500 (primers and probes listed in Table 5. Master mix components listed in Table 5.)

A. Cycling Conditions

Reverse transcription: 50°C for 3000 sec
Activation: 95°C for 900sec
Cycling: 50 cycles of 95°C for 10sec, 53°C for 25sec, 62°C for 70sec with optics on

B. Reaction Set-Up

e. Thaw primer solutions, probe solutions, and buffer mix and place them in bench top cool block in reagent hood.
f. Vortex for 5-10 sec at setting 7-10, then pop spin centrifuge 3-5 seconds to settle the liquid to the bottom of the tube. Place in ice or bench top cooler.

g. Prepare Master Mix for all sample and control reactions. *Keep all thawed components, reagents, controls and master mixes in cooling block.

h. Proceed to template hood and thaw IAC RNA and sample RNA in Template Hood.

i. Briefly spin the tubes 3-5 seconds in a POP micro-centrifuge to settle the liquid at the bottom of the tube.

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

k. Add <u>22 µl Master Mix</u> to each sample well.

l. Add 3µl of negative control (PCR water) to the designated well. *Note: in an effort to prevent cross contamination, it is recommended that after adding the negative control, you cover the plate with ABI plate cover and lift the cover as you add the template*

m. Add 3µl of sample template to the designated wells

n. Add 3µl of positive control template to the designated well.

o. Close cover plate briefly spin in 96 well plate spinner to bring down reagents to bottom of wells.

C. Instrument Set-Up

e. Place 96 well plate in ABI instrument and create run. Name the run with the assay, sample number, analysts initials.

f. Open ABI Software and Click on Icon for Advanced Setup

g. Fill out the "Experiment Name" field (other fields are optional)

h. Select 7500 (FAST) or 7500 depending on which machine you are using

i. Select “Quantitation Standard Curve”

j. Select “TaqMan Reagents”

k. Select “Standard (~3 hours to complete run)”

l. Click “Plate Set Up”

m. Under the “Define Targets” area click on the “Add New Target” until you have 3 targets

n. Fill target 1 with NoV GI, Target 2 with NoV GII, and Target 3 with IAC

o. 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)

p. Under Sample section click “Add Samples” until you have the requisite number of samples

Note this is not the amount of samples, but rather the total samples you have. For example if you have 2 samples in triplicate you have 2 samples under this area NOT 6

q. Fill in your samples names in each of the areas provided

r. Click “Assign Targets & Samples” tab

s. In the plate layout area highlight the locations where you have samples, while area is highlighted check all three targets located in the target area to the left of the plate layout.

t. Highlight each plate area with a single sample and check the sample located under the “assign samples to the selected well(s)” area located to the left of the plate layout until all samples are labeled

u. Under “Select the dye to use as the passive reference” scroll to FAM

v. Select “Run Method”

w. Change the reaction volume per well to 25 µl

x. Click tabular view

y. You will need 2 holding stages and 1 cycling stage with 3 steps (add or remove stages and steps as needed)

z. In the first holding stage set the parameters at 3000 seconds (50 minutes) at 50°C

aa. In the second holding stage set the parameters at 900 seconds (15 minutes) at 95°C

bb. In the cycling stage set the repeats to 50 cycles

cc. 1st stage set at 10 seconds at 95°C

dd. 2nd stage set at 25 seconds at 53°C (make sure you unclick the icon for collecting data)

ee. 3rd stage set at 70 seconds (1 minute; 10 seconds) at 62°C (click the icon for collecting data at "collect data on hold")

ff. Save the run method – Save as NoV Multiplex

(after you have saved the method you will no longer have to set it up, you may now simply select "Open Run Method" and select the saved method. However, you will have to click "Yes" on the pop up box)

gg. Click the "Analysis" tab on the right

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

ii. Click on each target so that it is highlighted

jj. For Cy5 (GI) – uncheck the "Use Default Settings" to the right under "CT Settings for Target 1"

kk. Uncheck "Automatic Threshold" – set Threshold to 0.1

ll. Uncheck "Automatic Baseline" – set "Baseline Start Cycle" to 3 and "End Cycle" to 10

mm. For TxR (Internal Control) – Repeat as for Cy5

nn. For Cy3 (GII) – Set Threshold to 0.01. Baselines are the same for other targets

oo. Click "Apply Analysis Settings"

pp. Click “Run” tab on the right

qq. Click green “Start Run” box

rr. Pop up screen will make you save the data. Save the data to your designated files.

Data Interpretation for Detection of NoV

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.

1. 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.
2. 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

3. 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, the RT-qPCR assay must be repeated.
   b. The RT-qPCR positive control is negative for GI and/or GII
   c. The average of the IAC Ct values for the sample replicates are more than 4.0 Ct’s greater than the Negative Control IAC Ct value, the RT-qPCR assay must be repeated using remaining RNA or RNA from a newly extracted saved tube with a 1µl RT-qPCR reaction in triplicate. If the repeat of the newly extracted sample yields average IAC Ct values 4.0 Cts higher than the Negative Control IAC Ct value, the sample must be repeated from the beginning using additional food sample. with the new repeat, the tubes will be split into 5 tubes and complete RT-qPCR with 1ul reactions in triplicate.

Table 5. Primer and Probe Sequences for NoV and Internal Amplification Control RNA

COG1R[a,∞]	5' CTT AGA CGC CAT CAT CAT TYA C 3'	5350-5371
COG2F[a,β]	5' CAR GAR BCN ATG TTY AGR TGG ATG AG 3'	5003-5028
COG2R[a,β]	5' TCG ACG CCA TCT TCA TTC ACA 3'	5080-5100
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' AGA TYG CGA TCY CCT GTC CA 3' -IB-RQ*	5317-5336
COGP1b[a,∞]	Cy5- 5' 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 6. ABI 7500 Amplification Reaction Components for NoV

Reagent	Initial Concentration	Volume per 25 µl reaction	Final Concentration
RNase Free H20		7.3 µl l	-
5 X OneStep RT-PCR Buffer	5X	5.0 µl	1 X
MgCl2~	50mM	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	0.15µM
OneStep RT-PCR Enzyme Mix		1.00 µl	
Superase·in	20 Units/µl	0.25 µl	5 Units
FAM ref dye	500nM	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 PCR cycles 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 4.0mM MgCl