Concentration, Extraction, and Detection of Norovirus and Hepatitis A virus in Soft Fruit

Produce contaminated with enteric viruses is the second leading cause of foodborne associated outbreaks in the US (CSPI, 2015). From 2013 to date, enteric viruses, notably hepatitis A virus (HAV) and norovirus (NoV) have been implicated in viral gastrointestinal illness associated with soft fruit in the US. 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 soft fruit (fresh/raw or frozen). 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.

4. Materials, Reagents, Equipment, and Supplies

a. 95% Ethanol (Sigma E7023)
b. Beef extract powder (Sigma Aldrich B4888)
c. DNase/RNase free water (Applied Biosystems molecular grade AM9937 or equivalent)
d. HCl (3N and 6N) and NaOH (2.5M and 5M) for titrating
e. RNA extraction silica gel kit (e.g., Qiagen QIAamp Viral RNA Mini Kit #52904)
f. Extech pH meter (Cole Parmer EW 53026-54)
g. NaCl (Sigma S3014 or equivalent)
h. KCl (Sigma P9541 or equivalent)
i. KH2PO4 (Sigma P9791 or equivalent)
j. Na2HPO4 (Sigma S5011 or equivalent)
k. Chloroform (Sigma C2432 or equivalent)
l. Tris Base (Fisher Scientific BP152-1 or equivalent)
m. Ethanol (95-100%)
n. Potassium acetate (Sigma P1190)
o. Pectinase (Fisher Scientific ICN19897910 from Aspergillus niger or equivalent)
p. Biological Safety Cabinets (BSC- 2 Type A2 or higher air exchange rate)
q. -70˚C or -80 ˚C Ultra low freezer
r. RNase-free latex or nitrile gloves
s. Vortex mixer
t. Pop micro-centrifuge

u. Whirl Pak filter bags 6x9 inch (Fisher Scientific 01-812-69 or equivalent)DNase/RNase-free microcentrifuge tubes, non-stick, low retention, siliconized 0.5ml (Life Technologies AM12350)
v. DNase/RNase-free microcentrifuge tubes 1.5 mL, non-stick, low retention, siliconized (Life Technologies AM12450)
w. Ice Bucket and ice, or bench top cooler
x. RNase Away® or equivalent
y. Smart Cycler tube holder
z. Smart Cycler cold block for Smart cycler tubes (-20 ºC)
aa. Adjustable Micropipettors (0.2 – 1000 µl), dedicated for RNA work only
bb. Filter barrier aerosol resistant micropipettor tips DNase/RNase free (0.2 – 1000 µl)
cc. Vortex Mixer
dd. Smart Cycler Reaction Tubes, 25-µl capacity (Fisher Scientific 11-400-3)
ee. Latex or nitrile gloves- Powder Free
ff. Cepheid Smart Cycler II
gg. Qiagen QIAamp Viral RNA Mini Kit (Cat no. 52904)
hh. Qiagen QIAshredder (Cat no.79654)
ii. Qiagen collection tubes (Cat no. 19201)
jj. OneStep™ PCR Inhibitor Removal Kit (Zymo Research D6030)
kk. ABI 7500
ll. Smart Cycler tube mini-centrifuge with Smart Cycler adapter
mm. ABI 96 well plates (Life Technologies) cat # 4346906
nn. ABI plates cover (Life Technologies) cat #4311971
oo. Mini Plate Spinner (Fisher Scientific)
pp. Pop micro-centrifuge
qq. 96 well cool rack (Sigma Aldrich #Z606634-1EA) or equivalent
rr. 500ml Blender (Cole Parmer EW 04243-25) or equivalent
ss. Blender Base (Fisher 14-509-19)
tt. 50ml Falcon tubes (Fisher 14-959-49A)
uu. 2.0ml microcentrifuge tubes DNase/RNase free (USA Scientific 1620-2799)
vv. 150 x 50 Petri Dishes (Fisher 08-757-148)
ww. Extech pH meter (Cole Parmer EW 53026-54)
xx. Centrifuge with applicable speeds of ≤12,000 x g, and with rotors capable of holding 50ml conical tubes
yy. 70ml ultracentrifuge tubes (Fisher NC 9959232)
zz. Ultracentrifuge with applicable speeds of ≥170,100 x g and with rotors capable of holding 70ml polypropylene ultracentrifuge tubes.
aaa. Microcentrifuge and DNase/RNAse free microcentrifuge tubes (Applied Biosystems AM12350)1.5 ml (AM12450) 2.0ml (12475)
bbb. Disposable transfer pipettes (Fisher 13-711-22)
ccc. -70°C freezer
ddd. glycine (Sigma G-7126)

eee. 150 x15 mm petri dishes (Fisher 08-757-148)
fff. PBS tissue culture grade (Sigma P5493) 10X solution to be diluted to 1X
ggg. Primer TE

hhh. Qiagen OneStep RT-PCR Kit; Cat No. 210210 (25 reactions) or 210212 (100 reactions)
iii. Ambion Superase·In (20 units/µl); Life Technologies Cat No. AM2694 (2,500 U) or AM2696 (10,000 U) Life Technologies
jjj. Orbital shaker (Fisher Scientific 11-676-231 or equivalent)
kkk. 50 mM MgCl2 or 25 mM MgCl2
lll. Internal Control RNA (available from BioGX Cat No. 750-0001)
mmm. 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)
nnn. negative RT-qPCR control (Nuclease free-water Applied Biosystems AM9937)
ooo. Fluorescein calibration dye (BioRad cat#1708780)
ppp. Extraction control murine norovirus (ATCC PTA-5935)

5. Concentration and Extraction Procedure

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

Remove pectinase from refrigerator and allow to come to room temperature

2.Add 100 µl extraction control (MNV) to sample (MNV preparation details described in BAM 26B).

3.Add 30 ml of 0.05 M Glycine/Tris/6% Beef Extract Buffer, pH 9.5 (Appendix A).

4.Add 50µl of room temperature pectinase (Appendix A).

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 PCR 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

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 ºC.

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

11. Bring total volume up to 65ml or 125g total weight (includes bottle and cap) with addition of tc PBS (Appendix A).

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

13. Centrifuge at 170,000 x *g* (37,000 rpm) for 45 min at 4°C (37,000rpm is specific for F40L rotor).

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

14. Slowly decant supernatant.

Discard excess liquid with disposable transfer pipette. 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 tc 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 s ±5 s.

18. Add 800 µl of chloroform to tube.

19. Vortex for 60 s ± 5 s.

20. Centrifuge at 3,000 x g for 5 min at 4°C.

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

Use one of the tubes for RNA extraction. Store other tubes at -70° C.

3. RNA Extraction

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

1. Add 560 µl prepared Buffer AVL with carrier RNA to tube from line 21.

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

2. Vortex for 20 sec and incubate at room temperature (15-25°C) for 10 min.

3. Add 100µl 2M Potassium Acetate (Appendix A) and invert 3 times.

4. Incubate on ice for 15 min.

5. Centrifuge for 10 min at 4°C at 14,000 rpm.

6. Carefully remove all of supernatant and transfer to 2ml DNase/RNase free. centrifuge tube (do not disturb pellet- if present)

7. Transfer 700 µl of supernatant to the QIAshredder column.

8. Centrifuge 2 min at maximum speed 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 another QIAshredder column, if necessary, until entire sample has been processed through the QIAshredder. Transfer the supernatant flow through to the 2.0 ml micro-centrifuge tube in step 9.

11. Add 700 µl of ethanol (95-100%) to the cleared lysate, 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* (≥8000 rpm) for 1 min. Place the QIAamp spin column in a new collection tube. Discard flow through and collection tube.

14. Continue to add sample 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. 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.

17. 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.

18. 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.

19. 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.

20. Place tube with RNA (step 19) on ice to prepare Zymo columns.

21. Prepare 2 Zymo columns per manufactures instructions.

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

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

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

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

26. Proceed with RT-qPCR or freeze at -80°C for long term storage.

4. Buffer and Reagent Recipes

a. 0.25 M Glycine, 0.05 M Tris/ 6% Beef Extract (pH 9.5)

Glycine (Sigma G-7126 or equivalent)	3.75 g
Beef Extract	60.0 g
Tris Base	12.0 g
Distilled Water	800 ml H_20
q.s. with distilled water to 1 liter	
pH to 9.5	
Sterilize 121°C for 15 min and store at 4 °C	

b. 2M Potassium Acetate

Potassium Acetate (Sigma P1190 or equivalent)	39.26 g
Distilled Water	150 ml

q.s. with distilled water to 200 ml
Sterilize 121°C for 15 min and store at room temperature

c. Pectinase

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

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

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

10X PBS	100 ml
Sterile distilled water	900 ml

Store at 4°C

e. 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.

f. 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.

g. 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

h. 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

i. 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 be demonstrate detection of MNV.

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

2. 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 or if the IAC is negative
   b. The RT-qPCR positive control is negative for MNV
   c. The MNV RT-qPCR is negative is 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)

E. 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.

F. 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

G. 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.

H. 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 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. 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 $MgCl_2$

I. 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.

J. 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

K. 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.

L. 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.

RT-qPCR Assays ABI 7500

4. 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 (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 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 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 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. Click tabular view

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

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

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

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

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

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

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

aa. 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)

bb. Click the "Analysis" tab on the right

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

dd. Click on each target so that it is highlighted

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

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

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

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

ii. Click "Apply Analysis Settings"

jj. Click "Run" tab on the right

kk. Click green "Start Run" box

ll. 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).

1. 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

2. 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.

3. 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. elect 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. Click tabular view

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

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

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

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

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

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

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

aa. 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)

bb. Click the "Analysis" tab on the right

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

dd. Click on each target so that it is highlighted

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

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

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

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

ii. Click "Apply Analysis Settings"

jj. Click "Run" tab on the right

kk. Click green "Start Run" box

ll. 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 H20		9.05µl	-
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
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 MgCl2

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

1. **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

2. **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 pop spin centrifuge 3-5 seconds to settle the liquid to the bottom of the tube. Place in ice or bench top cooler.

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

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

e. Briefly spin the tubes 3-5 seconds in a POP 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 designated wells

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

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

3. **Instrument Set-Up**

a. Place 96 well plate in ABI instrument and create run. Name the run with the assay, sample number, analysts 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 3 targets

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

k. 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)

l. 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

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. Click tabular view

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

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

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

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

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

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

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

aa. 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)

bb. Click the “Analysis” tab on the right

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

dd. Click on each target so that it is highlighted

ee. For Cy5 (GI) – uncheck the “Use Default Settings” to the right under “CT Settings for Target 1”

ff. Uncheck “Automatic Threshold” – set Threshold to 0.1
gg. Uncheck “Automatic Baseline” – set “Baseline Start Cycle” to 3 and “End Cycle” to 10

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

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

jj. Click “Apply Analysis Settings”

kk. Click “Run” tab on the right

ll. Click green “Start Run” box

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

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 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