Concentration, Extraction, and Detection of Norovirus and Hepatitis A virus in Molluscan Shellfish

Enteric viruses are the leading cause of foodborne infectious diseases in the United States and of the 9.4 million annual estimated cases of known foodborne illnesses, 58% are attributed to human noroviruses (NoV). Due to its severity, hepatitis A virus (HAV) is also a concern. This method described was developed for the concentration, extraction, and detection of enteric viruses from shellfish. This protocol will provide concentration and extraction of enteric viruses from whole shellfish (live shell stock or frozen). The concentration and extraction uses ultracentrifugation and Qiagen® RNeasy 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 Smart Cycler® 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. 1L sterile deionized/distilled/chilled water
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 RNeasy mini kit 74104)
f. chloroform (Sigma C2432)
g. pH strips (range 2-9 with 0.5 graduation) (Fisher Scientific 88-841
h. 50ml conical tubes (Fisher Scientific 50-550-489)
i. DNase/RNase-free microcentrifuge tubes, non-stick, low retention, siliconized 0.5ml (Life Technologies AM12350)
j. DNase/RNase-free microcentrifuge tubes 1.5 mL, non-stick, low retention, siliconized (Life Technologies AM12450)
k. Ice Bucket and ice, or bench top cooler
l. Smart Cycler tube holder
m. Smart Cycler cold block for Smart cycler tubes (-20 ºC)
n. Adjustable Micropipettors (0.2 – 1000 µl), dedicated for RNA work only
o. Filter barrier aerosol resistant micropipettor tips DNase/RNase free (0.2 – 1000 µl)
p. Vortex Mixer
q. Smart Cycler Reaction Tubes, 25-µl capacity (Fisher Scientific 11-400-3)
r. Latex or nitrile gloves- Powder Free

s. Cepheid Smart Cycler II
t. ABI 7500
u. Smart Cycler tube mini-centrifuge with Smart Cycler adapter
v. ABI 96 well plates (Life Technologies) cat # 4346906
w. ABI plates cover (Life Technologies) cat #4311971
x. Mini Plate Spinner (Fisher Scientific)
y. Pop micro-centrifuge
z. 96 well cool rack (Sigma Aldrich #Z606634-1EA) or equivalent
aa. 500ml Blender (Cole Parmer EW 04243-25) or equivalent
bb. Blender Base (Fisher 14-509-19)
cc. 50ml conical tubes (Fisher 14-959-49A or equivalent)
dd. 2.0ml microcentrifuge tubes DNase/RNase free (USA Scientific 1620-2799)
ee. 150 x 50 Petri Dishes (Fisher 08-757-148)
ff. Extech pH meter (Cole Parmer EW 53026-54)
gg. Centrifuge with applicable speeds of ≤10,000 x g, and with rotors capable of holding 50ml conical tubes
hh. 70ml ultracentrifuge tubes (Fisher NC 9959232)
ii. Ultracentrifuge with applicable speeds of ≥170,100 x g and with rotors capable of holding 70ml polypropylene ultracentrifuge tubes.
jj. FY14 50 carbon fiber rotor (ThermoFisher Scientific 46922 or equivalent)
kk. Microcentrifuge and DNase/RNAse free microcentrifuge tubes (Applied Biosystems AM12350)1.5 ml (AM12450) 2.0ml (12475)
ll. Disposable transfer pipettes (Fisher 13-711-22)
mm. -70°C freezer
nn. glycine (Sigma G-7126)
oo. threonine (Sigma T-8375)
pp. 150 x15 mm petri dishes (Fisher 08-757-148)
qq. PBS tissue culture grade (Sigma P5493) 10X solution to be diluted to 1X
rr. Disposable scalpels (Fisher Scientific 12 460 456)
ss. Guanidine isothiocynate (Fisher Scientific 15535-016)
tt. Primer TE (formula provided)

uu. Qiagen OneStep RT-PCR Kit; Cat No. 210210 (25 reactions) or 210212 (100 reactions)
vv. NaCl (Sigma S3014 or equivalent)
ww. KCl (Sigma P9541 or equivalent)
xx. KH2PO4 (Sigma P9791 or equivalent)
yy. Na2HPO4 (Sigma S5011 or equivalent)
zz. 50 mM MgCl2 or 25 mM MgCl2
aaa. Internal Control RNA (available from BioGX Cat No. 750-0001)
bbb. 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)
ccc. negative RT-qPCR control (Nuclease free-water Applied Biosystems AM9937)
ddd. Fluorescein calibration dye (BioRad cat#1708780)

eee. Extraction control murine norovirus (ATCC PTA-5935)
fff. Sterile milli-Q or DI water
ggg. Mini Plate Spinner (Fisher Scientific)
hhh. Pop micro-centrifuge
iii. 96 well cool rack (Sigma Aldrich #Z606634-1EA) or equivalent
jjj. 2.0ml microcentrifuge tubes DNase/RNase free (USA Scientific 1620-2799)

kkk. 70ml ultracentrifuge tubes (Fisher NC 9959232)
lll. Ultracentrifuge with applicable speeds of ≥170,100 x g and with rotors capable of holding 70ml polypropylene ultracentrifuge tubes.
mmm. Microcentrifuge and DNase/RNAse free microcentrifuge tubes (Applied Biosystems AM12350)1.5 ml (AM12450) 2.0ml (12475)
nnn. Disposable transfer pipettes (Fisher 13-711-22)

ooo. Primer TE

ppp. Qiagen OneStep RT-PCR Kit; Cat No. 210210 (25 reactions) or 210212 (100 reactions)
qqq. Ambion Superase·In (20 units/µl); Life Technologies Cat No. AM2694 (2,500 U) or AM2696 (10,000 U) Life Technologies
rrr. 50 mM MgCl2 or 25 mM MgCl2
sss. Internal Control RNA (available from BioGX Cat No. 750-0001)
ttt. 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)
uuu. negative RT-qPCR control (Nuclease free-water Applied Biosystems AM9937)
vvv. Fluorescein calibration dye (BioRad cat#1708780)
www. Extraction control murine norovirus (ATCC PTA-5935)

5. Concentration and Extraction Procedure

Concentration and extraction of norovirus and hepatitis A virus from shellfish

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

1. Place 12 shucked whole oysters (minus the adductor muscle that is left attached to the shell) in the Petri dish and dissect out the digestive gland.
   Note—if samples are frozen, allow thawing prior to dissecting

2. In a separate petri dish, mince the digestive glands and weigh a 4 ± 0.5 g in a clean sterile 50ml conical tube for analysis.
3. Add 100µl extraction control
4. Add 40ml sterile DI or Milli-Q water (water volume added should equal to 10 times weight of oyster meat).
5. Gently mix and transfer entire contents to blender and homogenize for 45 ± 15 s, ensuring that the sample is completely homogenized.
6. Pour homogenate into the same 50ml conical used above.
7. Adjust the pH of the homogenate to 4.5 ± 0.5 with the addition of HCl (3 N or 6 N) Check pH using a pH meter or strips (if using hand held pH meter, add 2 to 3 drops of homogenate to a clean petri dish and check pH—do not add contents back to sample).
8. Centrifuge the homogenate at 2,000 x g for 15 min at 4º C.
9. Decant and discard supernatant (viruses are should be adsorbed to the shellfish pellet at this point).
10. Resuspend pellet with 40ml of 0.75 M glycine/0.15 M NaCl (pH 7.6) and shake vigorously to bring into solution.
11. Adjust the pH of the homogenate to 7.5 ± 0.3 with the addition of NaOH (2.5 M or 5M) Check pH using a pH meter or strips (if using hand held pH meter, add 2 to 3 drops of homogenate to a clean petri dish and check pH—do not add contents back to sample).
12. Centrifuge the homogenate at 5,000 x g for 15 min at 4º C.
13. Collect supernatant/eluate into 70ml ultracentrifuge tube and store tube on ice.
14. Resuspend pellet in 20 ml of 0.5 M threonine/0.15 M NaCl (pH 7.5). Shake vigorously to bring into solution.
15. Centrifuge the homogenate at 5,000 x g for 15 min at 4º C.
16. Collect supernatant/eluate and combine into same 70ml ultracentrifuge tube from step 13.
17. Weigh and balance ultracentrifuge tubes with caps and contents to within 0.05 g of each other using PBS t.c .The weight of each tube with contents should be ±0.05 g (e.g. 65.05, 65.01, 65.03, 65.00 etc).
18. After balancing, spin for 1 hr at 170,000 x g.
19. Discard supernatant and resuspend pellet in 5 ml of PBS t.c . Transfer suspension to 50

ml conical tube. **(a gentle tap or vortexing on the side of the tube should dislodge pellet. At this point, the pellet and contents can be poured into the 50ml conical tube**).

20. Add 5 ml of chloroform to the suspension, close tightly and vortex for 45 ± 15 s to dissolve suspension.
21. Centrifuge at 1,700 x g for 15 min at 4°C.
22. Using a pipette, **carefully** collect the upper aqueous layer and transfer it to a clean ultracentrifuge tube—do not discard remaining sample. **(*store tube with contents on ice).**
23. Add 5 ml of 0.5 M threonine/0.15 M NaCl, pH 7.5 to the remaining sample containing the chloroform interface fraction, close tightly, and vortex for 30 s.
24. Centrifuge at 1,700 x g for 15 min at 4°C.

25. Using a pipette, **carefully** collect the top layer of the sample and combine with upper aqueous layer from step 22 into ultracentrifuge tube**.**
26. Discard remaining chloroform/sample as hazardous waste.
27. Add an additional 50 ml of PBS t.c. to sample in ultracentrifuge tube **(this should bring the total sample volume to ~ 60ml).**
28. Weigh and balance ultracentrifuge tubes with caps and contents to within 0.05 gof each other using PBS t.c .The weight of each tube with contents should be ±0.05 g (e.g. 65.05, 65.01, 65.03, 65.00 etc).

> ***Note: this can be a stopping point in the protocol. Once the tubes are balance and placed in the rotor with vacuum on, the samples can be left overnight at 4°C in the centrifuge and proceed with 1 hr spin immediately the next day.***

29. Centrifuge at 170,000 x g for 1 hr at 4^{o}C.
30. Discard supernatant (let tube settle for 1 min and discard excess liquid with disposable micro-pipettor)
31. Add 400 µl of PBS t.c to ultracentrifuge tube
32. Re-suspend pellet using a transfer pipette and evenly distribute into two clean DNase/RNase free microcentrifuge tubes.
33. Store concentrates at -70°C or proceed directly to RNA extraction

6. RNA Extraction

1. Obtain one concentrate.
2. Add 500 µl 6M GITC.
3. Vortex 60 ± 30 s dissolve concentrate (typically takes 60-90 s).
4. Add 700 µl of 50% EtOH and invert twice.
5. Pipette 700 µl of sample into an RNeasy mini spin column.
6. Centrifuge 10,000 x g for 1 min at room temperature.
7. Place column in new collection tube and discard flow through.
8. Add remaining sample to column.
9. Centrifuge 10,000 x g for 1 min at room temperature.
10. Place column in new collection tube and discard flow through .

11. Add 700 µL RW1 buffer to spin column and incubate for 15 min at room temperature.

Note: Heat primer TE to70°C (minimum 10 min-heating prior to elution)

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

Note: incubation not required at this step

17. Centrifuge at full speed (16,000 X g) for 2 minutes at room temperature.
18. Transfer column to new collection tube and centrifuge at room temperature full speed for 1 minute to dry column.
19. Carefully transfer column to 1.5ml low-retention/siliconized RNase/DNase free microcentrifuge tube.
20. Pipette 50 µL heated Primer TE onto silica-gel membrane of column.
21. Centrifuge at 10,000 x g for 1 min at room temperature.
    Note: Material that passed through column contains the viral RNA being isloated and is in the collection tube in which the cartridge was place
22. Pipette and additional 50 µL of heated Primer TE onto silica-gel membrane of column Pipette the eluted RNA back into column (~50 µL).
23. Centrifuge at 10,000 x g for 1 min at room temperature.
24. Proceed with RT-qPCR or freeze RNA at -70^{o}C.

7. Buffer and Reagent Recipes

a. 0.75 M Glycine/0.15M NaCl (pH 7.6)

Glycine (Sigma G-7126)	56.3 g
NaCl	8.8 g

Distilled water to make 1 L. Adjust pH to 7.576, autoclave and store at 4°C.

b. 0.5 M Threonine/0.15M NaCl (pH 7.5)

DL-Threonine	59.6 g
NaCl	8.8 g

Distilled water to make 1 L. Adjust pH to 7.5, autoclave and store at 4°C.

c. 6M Guanidine Isothiocyanate (GITC)

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

Measure 7.1 gram of GITC with a sterile container and sterile techniques. Add 4.5 ml of sterile distilled water to the container. A total volume of 10ml of GITC solution should

be noted. **Store at room temperature in dark or in black DNase/RNase free tubes. Solution stable for 1 month.**

d. 50% Ethanol

100% ethanol	5ml

Sterile distilled water to make 10ml
Store at room temperature

e. PBS (tissue culture grade)

10X PBS	100 ml
Sterile distilled/DI water	900 ml

Prepare in a clean sterile container 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

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

1. Thaw primer solutions, probe solutions, and buffer mix and place them in bench top cool block in reagent hood.
2. 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.
3. Prepare Master Mix for all sample and control reactions. *Keep all thawed components, reagents, controls and master mixes in cooling block.
4. Proceed to template hood and thaw IAC RNA and sample RNA in Template Hood.
5. Briefly spin the tubes 3-5 seconds in a POP micro-centrifuge to settle the liquid at the bottom of the tube.
6. Add appropriate volume of IAC, (0.2 ul/ rxn) to Master Mix (keep cold); Vortex briefly & Pulse spin.
7. Add 22 µl Master Mix to each sample well.
8. 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*

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

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

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

3. **Instrument Set-Up**

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

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

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

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

5. Select "Quantitation Standard Curve"

6. Select "TaqMan Reagents"

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

8. Click "Plate Set Up"

9. Under the "Define Targets" area click on the "Add New Target" until you have 3 targets

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

11. 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)

12. 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

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

14. Click "Assign Targets & Samples" tab

15. 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.

16. 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

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

18. Select “Run Method”

19. Click tabular view

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

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

22. In the second holding stage set the parameters at 900 seconds (15 minutes) at 95°C
23. In the cycling stage set the repeats to 50 cycles

24. 1st stage set at 10 seconds at 95°C

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

26. 3rd stage set at 70 seconds (1 minute; 10 seconds) at 62°C (click the icon for collecting data at “collect data on hold”)

27. 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)

28. Click the “Analysis” tab on the right

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

30. Click on each target so that it is highlighted

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

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

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

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

36. Click “Apply Analysis Settings”

37. Click “Run” tab on the right

38. Click green “Start Run” box

39. 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