Sampling, Verification, Preparation, and Testing Method for the detection of Hepatitis A in Scallops and Finfish
Date: 9/4/2018

Please note, this document is intended to provide supplemental general information to private laboratories on how to perform the initial sample preparation for Scallops and Finfish. This document does not outline all the analytical method or worksheet requirements for packages being submitted for FDA review. **

Please refer to the current FDA Laboratory Manual, Volume III, Section 7 for comprehensive information on private laboratory package requirements and the review process: https://www.fda.gov/downloads/ScienceResearch/FieldScience/LaboratoryManual/UC M092191.pdf

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Sampling and Verification Recommendation Implementation Date: 5/1/2018

Concentration, Extraction and of Hepatitis A Virus by Ultra Centrifugation and QIAAMP Viral RNA Mini Kit From Scallops and Finfish. Protocol information provided from Gulf Coast Seafood Laboratory Work Instruction Version Dated 1/12/2018

PART I – Introduction

1. **Purpose**

   The purpose of this work instruction is to describe:
   - Sampling methods and procedure recommendation
   - Verification recommendations to ensure:
     - Method functions (without any adaptation) in the user's laboratory on matrices not included in the original method validation.
     - Method can detect and identify the analyte of interest
     - Performance of a method in the user's laboratory against the specifications of the method.
   - Ultracentrifugation Concentration/ Qiashredder/QIAamp Viral RNA extraction of norovirus and hepatitis A virus (HAV) from scallops (adductor muscle) and finfish meat. This method also uses murine

norovirus as an extraction control.
- The detection of hepatitis A virus (HAV) using ABI 7500 platform.
- The detection of the extraction control, murine norovirus (MNV), using ABI 7500 platform.

2. Scope/Policy

- The work instructions for Ultra Centrifugation Concentration/ Qiashredder/QIAamp Viral RNA extraction of norovirus and hepatitis A virus (HAV) from scallops (adductor muscle) and finfish meat apply to all analysts or researchers involved in the analysis of scallops or finfish for the detection of HAV.
- The work instructions for the detection of hepatitisA virus (HAV) using ABI 7500 platform apply to all analysts or researchers involved in the detection of HAV in samples.
- The work instructions for the RT-qPCR detection of murine norovirus using ABI 7500 platform

3. Responsibility

Analysts (other positions descriptions if outside ORA) will be responsible for sample preparation, analysis, and documentation.

4. Background

The method described in Part IV, was developed by CFSAN's Gulf Coast Seafood Laboratory as a matrix and platform extension to BAM 26B. The method provides a rapid concentration of HAV from scallops and finfish meat using murine norovirus as an extraction control. This method is to be used in conjunction with the RT-qPCR detection described in Part V, RT-qPCR Detection of Murine Norovirus Using ABI 7500 Platform and Part VI, "RT-qPCR Detection of Hepatitis A Virus Using ABI 7500 Platform". The method provides a RT-qPCR assay for the detection of HAV and murine norovirus with the inclusion of an internal amplification control (IAC). **Valid HAV sample results are contingent upon the successful detection of the MNV extraction control from the sample being tested.**

PART II – Sampling

5. Sample Collection

5.1. If possible, please collect three, 400gram (one pound is 454 g) sub samples of product per sample. If 400 grams are not available, the minimum amount to collect per sub-sample is 150 g. Each subsample should consist of 2 retail units of product.

5.2. Three subs per sample should be collected. Two 50g composite (25g from each of 2 retail units in the subsample) per subsample will be tested.

5.3. Please document the lot code and any other unique identifiers on the product labeling for each sample.

5.4. Please provide photos of the entire labeling of the box sampled, including any identifier of the manufacturer.

5.5. Collect all samples aseptically, per the current IOM. Sample temperature should be maintained throughout the collection and shipment process.

PART III- Verification and Spiking Recommendation

6. **Verification Scheme required for labs performing the FDA validated method or other validated approved method for the first time**

   6.1. 2 Uninoculated samples
   6.2. 2 low spikes (at or near LOD= 5PFU HAV/g)
   6.3. 2 medium spikes (10x LOD= 50 PFU HAV/g)
   6.4. All 50 g samples must include an extraction control

7. **Validation Requirements required for labs using a different non-validated method**

   If the laboratory choses to use a non-FDA method and is not using an AOAC Official Methods of Analysis approved method or other third party method validated against a US reference method through collaborative study as required by above FDA Recommendation, ISO 16140:2016 or AOAC's Appendix J with collaborative study (e.g., an AOAC RI method is not a fully validated method), but have validated it in its laboratory for the product being tested, the laboratory must submit the validation package with the PL package for FDA review. The method must achieve the same performance specs as the FDA regulatory method

PART IV- Concentration, Extraction, and Detection of Hepatitis A Virus by Ultra Centrifugation and QIAAMP Viral RNA Mini Kit From Scallops and Finfish

8. **Procedure**

   8.1. Materials and Equipment

   > Glycine (Sigma G-7126 or equivalent; TLC grade or better)
   > Nuclease free-water (Applied Biosystems AM9937 or equivalent)
   > NaCl (Sigma S3014 or equivalent)
   > KCl (Sigma P9541 or equivalent)
   > KH_2PO_4 (Sigma P9791 or equivalent)
   > Na_2HPO_4 (Sigma S5011 or equivalent)
   > Chloroform (Sigma C2432 or equivalent)
   > Tris Base (Fisher Scientific BP152-1 or equivalent)
   > Ethanol (95-100%)
   > Biological Safety Cabinets (BSC- 2 Type A2 or higher air exchange rate)
   > -70˚C or -80 ˚C Ultra low freezer

RNase-free latex or nitrile gloves
Vortex Mixer
Pop micro-centrifuge

PBS (tissue culture grade) formula provided or PBS tissue culture grade (Sigma P5493), 10X solution to be diluted to 1X
Calibrated pipettors dedicated for RNA virus extraction (Rainin PR10, 17008649; PR20, 17008650; PR100, 17008651; PR1000, 17008653 or equivalent set)
Qiagen QIAamp Viral RNA Mini Kit (Cat no. 52904)
Qiagen QIAshredder (Cat no.79654)
Qiagen Collection Tubes (Cat no. 19201)
Filter-Barrier aerosol resistant pipette tips (USA Scientific 1120-3810, 1120-8810, 1126-7810 or equivalent)
Bench top cooling block, pre-chilled to 4°C (preferable) or ice bucket with ice
Micro-centrifuge (Eppendorf 5145 D or equivalent)
Micro-centrifuge tubes, 1.5-1.7 ml, siliconized, certified DNase & RNase free (Life Technologies AM12450 or equivalent)
Micro-centrifuge tubes, 2.0 ml, siliconized, certified DNase & RNase free (Life Technologies AM12475 or equivalent)
High speed centrifuge (ThermoFisher Scientific 46910)
FY14 50 carbon fiber rotor (ThermoFisher Scientific 46922 or equivalent)
50ml conical tubes (Fisher Scientific 14-959-49A or equivalent)
Ultra Centrifuge (Sorval WX90 or equivalent)
Carbon Fiber Rotor F40L, 8 x 100 ml (Thermofisher 096-087057 or equivalent)
70 mL Polycarbonate ultracentrifuge tubes w/aluminum cap tubes for F40L rotor (Fisher Scientific 010-1333 or equivalent)
Shaker, orbital or side-to-side (Labsource S16 304 or equivalent)
Balance (sensitivity of 0.01 g)
One Step PCR Inhibitor Removal Kit (Zymo Research D6030)
Whirl Pak filter bags 6x9 inch (Fisher Scientific 01-812-69 or equivalent)

8.2. Virus concentration

8.2.1. Add 50 g ± 2 g of scallops or fin fish (cut in no larger than 1" cubes pieces, for finfish loins or fillets, obtain 50 g portion from the surface) to a Whirl-Pak filter bag.

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

8.2.3. Add 50 ml of 0.05 M Glycine/Tris Buffer and tightly close.

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

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

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

8.2.6. Centrifuge at 9,000 x g for 30 min at 4 ºC.

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

8.2.8.   Add 10 ml of tc PBS to each tube to bring volume up to ~60 ml.

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

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

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

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

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

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

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

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

8.3 RNA Extraction

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

8.3.1   Add 560 µl prepared Buffer AVL with carrier RNA to tube from line 8.2.15.

8.3.2   Incubate at room temperature (15-25°C) for 10 min.

8.3.3   Resuspend pellet by pipetting up and down and vortexing.

8.3.4   Transfer 700 µl of liquid to the QIAshredder column.

8.3.5   Centrifuge 2 min at 16,000 x g in a microcentrifuge.

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

8.3.7   Repeat steps 8.3.4-8.3.6 using another QIAshredder column, if necessary, until entire sample has been processed through the QIAshredder. Transfer the column flow through to the 2.0 ml micro-centrifuge tube in step 8.3.6.

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

8.3.9   Apply 630 µl of the solution to a QIAamp mini column.

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

8.3.11 Repeat steps 8.3.9 and 8.3.10 until the entire sample has been passed through the column, discarding the collection tube each time.

8.3.12 Transfer the QIAamp mini column into a new 2 ml collection tube. Add 500 µl Buffer AW1. Incubate for 10 min. Centrifuge 1 min at 8000 x *g*. Discard flow through and collection tube.

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

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

8.3.15 To elute RNA, transfer the QIAamp mini column in to a new 1.5 ml low retention/siliconized DNase/RNase free centrifuge tube. Add 50 µl of pre-heated (70 ºC) Buffer AVE directly onto the QiaAmp silca-gel membrane. Close the tube gently, and centrifuge for 1 min at 8000 x g.

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

8.3.17 Place tube with RNA (step 8.3.16) on ice to prepare Zymo column.

8.3.18 Prepare Zymo column by snapping off the base, loosening the cap, inserting into a collection tube, and spin at 8,000 x *g* for 3 min.

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

8.3.20 Transfer RNA from step 8.3.17 to prepared Zymo One Step RT-PCR inhibitor remover column.

8.3.21 Screw on cap loosely and spin at 8,000 x *g* for 1 min.

8.3.22 Proceed with RT-qPCR as described in Work Instructions 2 and 3 or freeze at -80°C for long term storage.

*Note: Recommend completion of RT-qPCR assay for MNV for each spike sample to insure valid results for the extraction control prior to completion of the HAV RT-qPCR assay.

9. Working Solutions

0.05 M Glycine/0.05 M Tris Base Buffer, pH 9.5

Glycine	3.75 g
Tris Base	12.0 g

QS to 1L with MilliQ (18 ohm) Adjust pH to 9.5, sterilize 121°C for 15 min and store at 4°C.

PBS (tissue culture grade)

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.

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

1M tris pH 8.0	100 µl
0.05M EDTA	20 µl

PCR-grade water (DNase/RNase free) 9.88 ml
Prepare in sterile 50ml conical tube. Store at room temperature

Fluorescein Calibration Dye 500nM stock solution

Fluorescein	0.5 µl
Primer TE	1000 µl

Mix well and store in dark tubes at 4°C for 2 months or 20°C for 4 months.

ALV 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. sample	Vol. Buffer AVL	Vol. carrier	No. sample	Vol. Buffer AVL	Vol. carrier
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

Part V- Work Instruction 3 RT-qPCR Detection of Murine Norovirus Using ABI 7500 Platform

RT-qPCR Assay Parameters Murine Norovirus

The RT-qPCR protocols for the ABI 7500 will require 25µl reactions. Three replicates per sample plus a positive and negative control will be run. Prior to RT-qPCR master mix preparation, protocols for ABI 7500 platforms should be set-up.

10. **Procedure**

10.1. **Materials, Equipment, and Supplies**

DNase/RNase-free microcentrifuge tubes, non-stick, low retention, siliconized 0.5ml (Life Technologies AM12350)

DNase/RNase-free microcentrifuge tubes 1.5 mL, non-stick, low retention, siliconized (Life Technologies AM12450)

Ice Bucket and ice, or bench top cooler

Adjustable Micropipettes (0.2 – 1000 µl), dedicated for RNA work only

Filter barrier aerosol resistant micropipettes tips DNase/RNase free sizes (0.2-1000 µl)

Vortex Mixer

Latex or nitrile gloves- Powder Free

ABI 7500

ABI 96 well plates (Life Technologies) cat # 4346906

ABI plates cover (Life Technologies) cat #4311971

Mini Plate Spinner (Fisher Scientific)

96 well cool rack (Sigma Aldrich #Z606634-1EA) or equivalent

Microcentrifuge and DNase/RNAse free microcentrifuge tubes (ABI AM12350)1.5 ml (AM12450) 2.0ml (12475)

Disposable transfer pipettes (Fisher 13-711-22)

-70˚C or -80 ˚C Ultra low freezer

Qiagen OneStep RT-PCR Kit; Cat No. 210210 (25 reactions) or 210212 (100 reactions)

50 mM MgCl2 or 25 mM MgCl2

Ambion Superase·In (20 units/µl); Life Technologies Cat No. AM2694 (2,500 U) or AM2696 (10,000 U) Life Technologies

Internal Control RNA (BioGX Cat No. 750-0001)

Positive controls (murine norovirus RNA—ATCC PTA-5935)

Negative RT-qPCR control (Nuclease free-water Life Technologies AM9937)

Fluorescein calibration dye (BioRad cat#1708780)

11.   Out lined MNV RT-qPCR Protocol ABI 7500

11.1. 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

11.2.   Reaction Set-Up

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

11.2.2. 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.

11.2.3. 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.

11.2.4. Proceed to template area and thaw IAC RNA and sample RNAin
Template Hood/Area.

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

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

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

11.2.8. 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, youcover
   the plate with ABI plate cover and lift the cover as you add the
   template*

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

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

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

11.3.   Instrument Set-Up

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

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

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

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

11.3.5. Select "Quantitation Standard Curve"

11.3.6. Select "TaqMan Reagents"

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

11.3.8. Click "Plate Set Up"

11.3.9. Under the "Define Targets" area click on the "Add NewTarget" unt i l you have 3 targets

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

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

11.3.12. Under Sample section click "AddSamples" 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

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

11.3.14. Click "Assign Targets & Samples" tab

11.3.15. 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.

11.3.16. 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

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

11.3.18. Select "Run Method"

11.3.19. Click tabular view

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

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

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

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

11.3.24. 1st stage set at 15 seconds at 95°C

11.3.25. 2nd stage set at 20 seconds at 55°C (make sure you unclick the icon for collecting data)

11.3.26. 3rd stage set at 60 seconds (1 minute) at 62°C (click the icon for collecting data at "collect data on hold")

11.3.27. 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)

11.3.28. Click the "Analysis" tab on the right

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

11.3.30. Click on each target so that it is highlighted

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

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

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

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

11.3.35. Click "Apply Analysis Settings"

11.3.36.   Click "Run" tab on the right

11.3.37.   Click green "Start Run" box

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

12. Supporting Documents

Qiagen One-Step RT-PCR kit handbook, October 2012 www.qiagen.com

13. Attachments

13.1 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 4.0 Ct's less that the
      Negative Control IAC Ct Value.

13.2 Amplification Reaction Components for MNV[∞]

Reagent	Initial Concentration	Volume per 25 µl reaction	Final Concentration
RNase Free H$_2$0		9.8 µl	-
5 X OneStep RT-PCR Buffer	5X	5.0 µl	1 X
MgCl$_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

RT-qPCR Assay Parameters Hepatitis A virus

The RT-qPCR protocols for the ABI 7500 will require 25µl reactions. Three replicates per sample plus a positive and negative controls will be run. Prior to RT-qPCR master mix preparation, protocols for ABI 7500 platforms should be set-up.

14. Procedure

    14.1.   **Materials, Equipment, and Supplies**

DNase/RNase-free microcentrifuge tubes, non-stick, low retention, siliconized 0.5ml (Life Technologies AM12350)
DNase/RNase-free microcentrifuge tubes 1.5 mL, non-stick, low retention, siliconized (Life Technologies AM12450)

Ice Bucket and ice, or bench top cooler
Adjustable Micropipettes (0.2 – 1000 µl), dedicated for RNA work only Filter barrier aerosol resistant micropipette tips DNase/RNase free sizes (0.2- 1000 µl)
Vortex Mixer
Latex or nitrile gloves- Powder Free ABI
7500
ABI 96 well plates (Life Technologies) cat # 4346906 ABI
plates cover (Life Technologies) cat #4311971 Mini Plate
Spinner (Fisher Scientific)
96 well cool rack (Sigma Aldrich #Z606634-1EA) or equivalent Microcentrifuge and DNase/RNAse free microcentrifuge tubes (ABI AM12350)1.5 ml (AM12450) 2.0ml (12475)
Disposable transfer pipettes (Fisher 13-711-22)
-70˚C or -80 ˚C Ultra low freezer
Qiagen OneStep RT-PCR Kit; Cat No. 210210 (25 reactions) or 210212 (100 reactions)
50 mM MgCl2 or 25 mM MgCl2
Ambion Superase·In (20 units/µl); Life Technologies Cat No. AM2694 (2,500 U) or AM2696 (10,000 U) Life Technologies
Internal Control RNA (BioGX Cat No. 750-0001) Positive
controls (HAV RNA— ATCC VR-1402
Negative RT-qPCR control (Nuclease free-water Life Technologies AM9937)
Fluorescein calibration dye (BioRad cat#1708780)

15. Out lined HAV RT-qPCR Protocol ABI 7500

15.1.   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

15.2.   Reaction Set-Up

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

15.2.2. 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.

15.2.3. 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.

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

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

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

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

15.2.8. 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*

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

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

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

15.3.   Instrument Set-Up

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

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

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

15.3.4.   elect 7500 (FAST) or 7500 depending on which machine you are using

15.3.5.   Select "Quantitation Standard Curve"

15.3.6.   Select "TaqMan Reagents"

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

15.3.8.   Click "Plate Set Up"

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

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

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

15.3.12.   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

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

15.3.14.   Click "Assign Targets & Samples" tab

15.3.15.   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.

15.3.16.   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

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

15.3.18. Select "Run Method"

15.3.19. Click tabular view

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

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

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

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

15.3.24. 1st stage set at 10 seconds at 95°C

15.3.25. 2nd stage set at 25 seconds at 53°C (make sure you unclick the icon for collecting data)

15.3.26. 3rd stage set at 70 seconds (1 minute; 10 seconds) at 64°C (click the icon for collecting data at "collect data on hold")

15.3.27. 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)

15.3.28. Click the "Analysis" tab on the right

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

15.3.30. Click on each target so that it is highlighted

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

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

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

15.3.34. Click "Apply Analysis Settings"

15.3.35.   Click "Run" tab on the right

15.3.36.   Click green "Start Run" box

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

16. Attachments

16.1 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 RT-qPCR 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 greater than 4.0 Ct, 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.

16.2 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