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

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Revision History: Chapter 26B has been archived and all sections have been updated and extended to include the RT-qPCR analysis of multiple food matrices for hepatitis A virus and norovirus GI and GII.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 Chapter 26A has also been archived.

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Appendix A: Validation Data for the Concentration, Extraction, and Detection of Norovirus Genogroup I, Norovirus Genogroup II, and Hepatitis A Virus from Green Onion and Leafy Greens

Green onions were artificially contaminated with three levels of HAV and the murine norovirus extraction control. In this method validation, 8 of the 10 participating FDA, CFSAN, or FERN laboratories produce acceptable data consistent with the FDA Guidelines for the Validation of Analytical Methods for the Detection of Microbial Pathogens in Foods, 1st Ed (2011) for a level 3 validation (Tables A1 and A2). The overall detection frequency of HAV from green onion spikes was 97% and 75% for the 50 pfu and 5 pfu/g test portions, respectively (Table A3).

For the leafy greens matrix, romaine lettuce was spiked with three levels of norovirus and HAV. This matrix extension produced acceptable data consistent with the FDA Guidelines for the Validation of Analytical Methods for the Detection of Microbial Pathogens in Foods and Feeds, 2nd Ed (2015). HAV and norovirus GII were detected in all replicates at all inoculum levels. In romaine lettuce, norovirus GI had a detection frequency of 80% and 60% in the 3 genome copies (g.c.)/g and 0.3 g.c./g inoculum levels (Table A4 A-B). In spinach, norovirus GI had a detection frequency of 47% and 60% at the 3 g.c./g and 0.3 g.c./g inoculum levels (Table A4 A-B).

Sample Preparation

Green onions were purchased from a local retail market. The test portions were cut in 2" and 5" segments and placed into Whirl-pak® bags. Samples were spiked and held at 4 °C for 3 days prior to shipment. Twenty test portions, in triplicate, were prepared and shipped in coolers with ice bricks to the participating laboratories by CFSAN's Moffett Center Institute of Food Safety and Health. Sample analysis was begun within 24 hrs of receipt.

Virus Inoculum

HAV inoculum used for seeding was the vaccine strain (HAV175/18f) propagated in house utilizing FrHK cell line. Murine norovirus (MNV) used for seeding was murine norovirus-1 propagated in house using RAW 264.7 cell line. Norovirus GI and GII from chloroform extracted clinical specimen were used for seeding samples. Three inoculation levels were used for HAV spikes; Low (5 PFU/g of HAV), High (50 PFU/g of HAV) and uninoculated. The norovirus spikes were 330 genomic copies for the high, 33 for the medium, and 0.3-3 genomic copies for the low inoculum. MNV was inoculated in all test portions at 4 x 10^3 PFU.

Table A1. Percentage of samples with expected results for each participating laboratory

	# correct/ # samples; % correct			
	High	**Low**	**Negatives**	**RT-qPCR Controls**
Lab #1	8 of 8; 100%	6 of 8; 75%	4 of 4; 100%	√
Lab #2	8 of 8; 100%	8 of 8; 100%	4 of 4; 100%	√
Lab #3	8 of 8; 100%	5 of 8; 63%	4 of 4; 100%	√
Lab #4	6 of 8; 75%	3 of 8; 38%	4 of 4; 100%	√
Lab #5	8 of 8; 100%	8 of 8; 100%	1 of 4; 25% *	√
Lab #6	8 of 8; 100%	8 of 8; 100%	4 of 4; 100%	√
Lab #7	8 of 8; 100%	7 of 8; 88%	4 of 4; 100%	√
Lab #8	8 of 8; 100%	3 of 8; 38%	4 of 4; 100%	√
Lab #9	3 of 4; 75% **	1 of 4; 25% **	3 of 3; 100% **	√ **
Lab #10	8 of 8; 100%	4 of 8; 50%	4 of 4; 100%	√

*Denotes data not valid/not reported

**Denotes data not valid/not reported

Table A2. Laboratory data from detection of HAV in green onion

Sample #	Key	Lab#1	Lab #2	Lab #3	Lab #4	Lab #5	Lab #6	Lab #7	Lab #8	Lab #9	Lab #10
1	-	-	-	-	-	+*	-	-	-	-	-
2	+, L	+	+	-**	+	+	+	+	+	+	+
3	+, L	-**	+	+	-**	+	+	+	-**	-**	+
4	+, H	+	+	+	-**	+	+	+	+	+	+
5	+, H	+	+	+	-**	+	+	+	+	+	+
6	-	-	-	-	-	+*	-	-	-	-	-
7	+, L	-**	+	+	+	+	+	-**	-**	-**	-**
8	+, H	+	+	+	+	+	+	+	+	-**	+
9	+, H	+	+	+	+	+	+	+	+	error	+
10	-	-	-	-	-	+*	-	-	-	error	-
11	+, H	+	+	+	+	+	+	+	+	error	+
12	+, L	+	+	-**	+	+	+	+	-**	error	+
13	+, L	+	+	+	-**	+	+	+	-**	error	-**
14	+, L	+	+	+	-**	+	+	+	-**	error	-**
15	+, H	+	+	+	+	+	+	+	+	error	+
16	+, H	+	+	+	+	+	+	+	+	error	+
17	-	-	-	-	-	-	-	-	-	-	-
18	+, L	+	+	+	-**	+	+	+	+	-**	-**
19	+, L	+	+	-**	-**	+	+	+	+	-**	+
20	+, H	+	+	+	+	+	+	+	+	+	+
Positives	+	+	+	+	+	+	+	+	+	+	+
Negatives	-	-	-	-	-	-	-	-	-	-	-

*Denotes lab data not valid/not reported

**Denotes false negatives

"Error" denotes data not valid/not reported/instrument error

Table A3. Detection Frequencies of HAV

50 PFU/g HAV- High Inoculum	97%
5 PFU/g HAV- Low Inoculum	75%
0 PFU/g – No Incoculum (Negative Control)[a]	8%
RT-qPCR Controls	
False negative/invalid RT-qPCR controls	0%
False positive/invalid RT-qPCR controls	0%

[a] Results derived from one laboratory; attributed to cross contamination

Table A4 (A-B). Matrix extension data for norovirus GI, GII, and HAV in leafy greens

A. Norovirus GI and GII

GI					
Leafy Green	**Medium^**	**Low***	**~Low/LOD**	**Uninoculated**	**MNV EC^**
Romaine Lettuce – Positive samples	5 of 5	12 of 15	3 of 5	0 of 5	30 of 30
Romaine Lettuce – Percent positive	100	80[∞]	60[∞]	0	100
Spinach – Positive samples	5 of 5	7 of 15	3 of 5	0 of 5	30 of 30
Spinach – Percent positive	100	47[∞]	60[∞]	0	100
GII					
Leafy Green	**Medium^**	**Low***	**~Low/LOD**	**Uninoculated**	**MNV EC^**
Romaine Lettuce – Positive samples	5 of 5	15 of 15	5 of 5	0 of 5	30 of 30
Romaine Lettuce – Percent positive	100	100	100	0	100
Spinach – Positive samples	5 of 5	15 of 15	5 of 5	0 of 5	30 of 30
Spinach – Percent positive	100	100	100	0	100

^ NoV GII 330 genomic copies per gram
* NoV GI\GII 33 genomic copies per gram
~ NoV GI\GII 0.3-3 genomic copies per gram
[^] MNV extraction control
[∞] Fractional positives

B. HAV

Leafy Green	High[β]	Medium[$]	Low [#]	Uninoculated	MNV EC^
Romaine Lettuce – Positive Samples	5 of 5	15 of 15	5 of 5	0 of 5	30 of 30
Romaine Lettuce – Percent Positive	100	100	100	0	100
Spinach – Positive Samples	5 of 5	15 of 15	5 of 5	0 of 5	30 of 30
Spinach – Percent Positive	100	100	100	0	100

[β] HAV 10 PFU per gram
[$] HAV 1 PFU per gram
HAV 0.1 PFU per gram
[^] MNV extraction control

Conclusion

The MLV of the HAV concentration, extraction, and detection method for green onion has demonstrated acceptable sensitivity and specificity for regulatory analysis of green onion samples. Data supporting the reliability of the real-time RT-qPCR detection assays for HAV and norovirus are provided in Appendix F and Appendix G, respectively. In addition, a matrix extension for the concentration and extraction of enteric viruses from leafy greens (romaine lettuce and spinach) has been completed using this method. The results show the method is sensitive, reproducible, and robust and has established the "Fitness of Purpose" for concentration, extraction, and detection of HAV, norovirus GI, and norovirus GII from leafy greens. The detection frequency of the concentration, extraction, and detection method was 97-100% for the medium and high inocula, and 47-100% for the low inocula, depending on the specific matrix/target combination. The internal amplification control demonstrated little to no inhibition (< 4 C_ts) for all detection assays. The limit of detection for this validated concentration, extraction, and detection method is 1-5 PFU/g.

Our conclusion from the MLV data is that the concentration, extraction, and detection method can be used for determination of HAV in green onion samples. The subsequent matrix extension demonstrates the concentration, extraction, and detection method can be used for HAV, norovirus GI, and norovirus GII from green onion and leafy greens. These assays are ready to be incorporated into the Bacteriological Analytical Manual and ongoing Office of Regulatory Affairs Field Assignments.

Appendix B: Validation Data for the Concentration, Extraction, and Detection of Norovirus Genogroup I, Norovirus Genogroup II, and Hepatitis A Virus from Soft Fruit: Fresh and Frozen

Sample Preparation

Template for the spikes consisted of chloroform extracted virus from clinical samples or cell culture lysate. QIAamp Viral RNA kit (Qiagen, Carlsbad, CA) was used to extract RNA from MNV and HAV materials and serve as positive controls. RNA transcripts created from cloning GI and GII strains were used as positive controls. All template and RT-qPCR reagents were prepared and stored at -20 °C until analysis.

Fresh/raw and frozen fruit samples were purchased from a local retail market. The test portions were weighed, in triplicate, and placed into Whirl-pak® bags. The test portions were spiked with norovirus GI, GII, and HAV and aged at 4 °C for fresh fruit and 20 °C for frozen fruit for 48 hrs. Prior to analysis, all test portions (after defrosting where applicable) were spiked with an aliquot of MNV extraction control.

Virus Inocula

The HAV inoculum used for seeding was the vaccine strain (HAV175/18f) propagated in house utilizing FrHK cell line. Murine norovirus (MNV) which served as the extraction control was propagated in house using RAW 264.7 cell line. Four inoculation levels were used for norovirus: low, medium, high, and uninoculated. For HAV, low and medium spikes were 0.1 and 1 PFU/g of HAV, respectively. The norovirus spikes were 330 genomic copies for the high, 33 for the medium, and 0.3-3 genomic copies for the low inoculum. MNV was inoculated in all samples at 10^3 PFU.

Results: Fresh and Frozen Fruit

The matrix extension for the concentration, extraction, and detection of norovirus GI, GII, and hepatitis A virus (HAV) in soft fruit was conducted at GCSL in accordance with a level 2 (Part-a) validation using Table 1 in the FDA Guidelines for the Validation of Analytical Methods for the Detection of Microbial Pathogens in Foods and Feeds, 2nd Ed (2015). Results of this validation are for 5 soft fruit matrices including blackberries, raspberries, strawberries, pomegranate arils, and mixed fruit (cantaloupe, pineapple, honeydew, watermelon, red grapes, and strawberries). Murine norovirus (MNV) was used as an extraction control. In addition, a 'negative process control' was inoculated with only the extraction control. For all fruit matrices, the extraction control was detected in all replicate test portions for the fresh and frozen fruits (Table B1). Norovirus inoculum for fresh/raw and frozen fruit had a detection frequency of 100% for the high inoculum (Table B2). For the raspberry, strawberry, and pomegranate frozen samples, there was a freezer malfunction that affected the norovirus GII spikes. Accordingly, test portions were reinoculated and analyzed separately; these are the data presented. The detection frequencies for the norovirus low inoculum ranged from 33-100% for the fresh/raw and 11-67% for the frozen (Table B2). The medium inoculum detection ranged from 94-100% for norovirus. There was no significant inhibition in any of the different fruit matrices except for the frozen pomegranate. The pomegranate RT-qPCR reactions were performed using the 3 µl template

followed by the 1 µl template volume as there was inhibition in the 3 µl reaction. The extraction control was detected in the 3 µl and 1 µl reactions.

The detection frequency for the medium inoculum of HAV was 100% for fresh/raw and frozen fruit matrices, except for the frozen strawberry, which had a detection frequency of 94% (Table B3). The extraction efficiencies averaged 59% for fresh/raw fruit and 30% for frozen fruit (Table B4). The LOD for norovirus ranged from 0.3-3 genomic copies per gram while HAV LOD was determined to be 0.1 PFU/gram. The average C_t values for each matrix showed 1 log difference in the norovirus detection for the high, medium, and low replicates. These results meet the criteria set forth by the FDA Guidelines for the Validation of Analytical Methods for the Detection of Microbial Pathogens in Foods, 2nd Ed (2015) for a matrix extension.

Table B1. Murine Norovirus Extraction Control Fresh and Frozen Fruit Data

Fruit	Raw/Fresh or Frozen	High	Medium	Low	Uninoculated
Blackberry – Percent positive	Raw/Fresh	100	100	100	100
Blackberry – Percent positive	Frozen	100	100	100	100
Raspberry – Percent positive	Raw/Fresh	100	100	100	100
Raspberry – Percent positive	Frozen	100	100	100	100
Strawberry – Percent positive	Raw/Fresh	100	100	100	100
Strawberry – Percent positive	Frozen	100	100	100	100
Pomegranate Arils – Percent positive	Raw/Fresh	100	100	100	100
Pomegranate Arils – Percent positive	Frozen	100	100	100	100
Mixed Fruit – Percent positive	Raw/Fresh	100	100	100	100
Mixed Fruit – Percent positive	Frozen	100	100	100	100

Table B2. Norovirus GI/GII Fresh/Raw and Frozen Fruit Data

Fruit	Raw/Fresh or Frozen	High^ 330 g/c	Medium* 33 g/c	Low~ 0.3-3 g/c	Uninoculated 0 g/c
Blackberry – Positive Samples	Raw/Fresh	9 of 9	18 of 18	8 of 9	0 of 9
Blackberry – Percent Positive	Raw/Fresh	100	100	88∞	0
Blackberry – Positive Samples	Frozen	9 of 9	18 of 18	3 of 9	0 of 9
Blackberry – Percent Positive	Frozen	100	100	33∞	0
Raspberry – Positive Samples	Raw/Fresh	9 of 9	18 of 18	6 of 9	0 of 9
Raspberry – Percent Positive	Raw/Fresh	100	100	67∞	0
Raspberry – Positive Samples	Frozen	9 of 9	18 of 18	6 of 9	0 of 9
Raspberry – Percent Positive	Frozen	100	100	67∞	0
Strawberry – Positive Samples	Raw/Fresh	9 of 9	18 of 18	9 of 9	0 of 9
Strawberry – Percent Positive	Raw/Fresh	100	100	100	0
Strawberry – Positive Samples	Frozen	9 of 9	17 of 18	1 of 9	0 of 9
Strawberry – Percent Positive	Frozen	100	94∞	11∞	0
Pomegranate Arils – Positive Samples	Raw/Fresh	9 of 9	18 of 18	3 of 9	0 of 9
Pomegranate Arils – Percent Positive	Raw/Fresh	100	100	33∞	0
Pomegranate Arils – Positive Samples	Frozen@	9 of 9	17 of 18	1 of 9	0 of 9
Pomegranate Arils – Percent Positive	Frozen@	100	94∞	11∞	0
Mixed Fruit – Positive Samples	Raw/Fresh	9 of 9	18 of 18	9 of 9	0 of 9
Mixed Fruit – Percent Positive	Raw/Fresh	100	100	100	0
Mixed Fruit – Positive Samples	Frozen	9 of 9	18 of 18	2 of 9	0 of 9
Mixed Fruit – Percent Positive	Frozen	100	100	22∞	0

^ NoV GII 330 genomic copies per gram of fruit
* NoV GI\GII 33 genomic copies per gram of fruit; data are reported as independent results for GI and GII (9 replicates each)
~ NoV GI\GII 0.3-3 genomic copies per gram of fruit; data are reported as detected if either GI or GII was detected in the replicate
∞ Fractional positives
@ Results for 1 µl reaction

Table B3. HAV Fresh/Raw and Frozen Fruit Data

Fruit	Raw/Fresh or Frozen	Medium 1 PFU/g	Low 0.1 PFU/g	Uninoculated 0 PFU/g
Blackberry – Positive Samples	Raw/Fresh	9 of 9	9 of 9	0 of 9
Blackberry – Percent Positive	Raw/Fresh	100	100	0
Blackberry – Positive Samples	Frozen	9 of 9	9 of 9	0 of 9
Blackberry – Percent Positive	Frozen	100	100	0
Raspberry – Positive Samples	Raw/Fresh	9 of 9	9 of 9	0 of 9
Raspberry – Percent Positive	Raw/Fresh	100	100	0
Raspberry – Positive Samples	Frozen	9 of 9	9 of 9	0 of 9
Raspberry – Percent Positive	Frozen	100	100	0
Strawberry – Positive Samples	Raw/Fresh	9 of 9	9 of 9	0 of 9
Strawberry – Percent Positive	Raw/Fresh	100	100	0
Strawberry – Positive Samples	Frozen	8 of 9	6 of 9	0 of 9
Strawberry – Percent Positive	Frozen	89[∞]	67[∞]	0
Pomegranate Arils – Positive Samples	Frozen	9 of 9	9 of 9	0 of 9
Pomegranate Arils – Percent Positive	Frozen[@]	100	100	0
Pomegranate Arils – Positive Samples	Raw/Fresh	9 of 9	8 of 9	0 of 9
Pomegranate Arils – Percent Positive	Frozen[@]	100	89[∞]	0
Mixed Fruit – Positive Samples	Raw/Fresh	9 of 9	9 of 9	0 of 9
Mixed Fruit – Percent Positive	Frozen	100	100	0
Mixed Fruit – Positive Samples	Raw/Fresh	9 of 9	9 of 9	0 of 9
Mixed Fruit – Percent Positive	Frozen	100	100	0

[∞] Fractional positives
[@] Results for 1 µl reaction

Table B4 (A-C). Extraction efficiency, LOD and LOQ for fresh and frozen soft fruit

A. Extraction Efficiency

	FRESH	FROZEN
MNV Overall Extraction Efficiency	59%	30%

B. LOQ (Limit of Quantification) – genomic copies (gc) or PFU/gram

	GI g.c./g	GII g.c./g	HAV PFU/g	GI g.c./g	GII g.c./g	HAV PFU/g
Blackberry	3	3	1	30	30	1
Raspberry	3	30	1	30	30	1
Strawberry	3	30	1	30	30	1
Pomegranate Arils	3	30	1	30	30	1
Mixed Fruit	3	3	1	30	30	1

C. LOD (Limit of Detection) – genomic copies (gc) or PFU/gram

	GI g.c./g	GII g.c./g	HAV PFU/g	GI g.c./g	GII g.c./g	HAV PFU/g
Blackberry	0.3	0.3	0.1	3	3	0.1
Raspberry	0.3	0.3	0.1	3	ND*	0.1
Strawberry	0.3	0.3	0.1	3	ND*	0.1
Pomegranate Arils	0.3	0.3	0.1	3	ND*	0.1
Mixed Fruit	0.3	0.3	0.1	3	3	0.1

* Not determined

Conclusion

The concentration, extraction, and detection of enteric viruses from soft fruit can be challenging due the nature of the acidity of the fruit and the propensity for enteric viruses to attach to surfaces in acidic conditions. The matrix extension validation of the norovirus and HAV concentration and extraction assay to fresh and frozen soft fruit demonstrated sensitivity and specificity for the detection of norovirus and HAV. The extraction control was detected in all replicates spiked, even those that exhibited inhibition. The data interpretation of the detection assay portion of this method includes recommendations on the next step to take should the sample exhibit inhibition (BAM Chapter 26, Sections B2 and B4), which was the case for the pomegranate arils. The detection frequency of the method was 100% for the high inoculum, 89-100% for the medium inoculum, and 11-100% for the low inoculum, depending on the specific matrix/target combination. The results demonstrate the method is sensitive, reproducible, and robust and has established the "Fitness of Purpose" for concentration, extraction, and detection of norovirus GI, norovirus GII, and HAV from soft fruit matrices.

Our conclusion from this matrix extension validation is that the method can be used for the concentration, extraction, and detection of norovirus and HAV from fresh and frozen soft fruit.

This protocol is ready to be incorporated into the Bacteriological Analytical Manual and ongoing Office of Regulatory Affairs Field Assignments.

Appendix C: Validation Data for the Concentration, Extraction, and Detection of Norovirus Genogroup I, Norovirus Genogroup II, and Hepatitis A Virus from Molluscan Shellfish

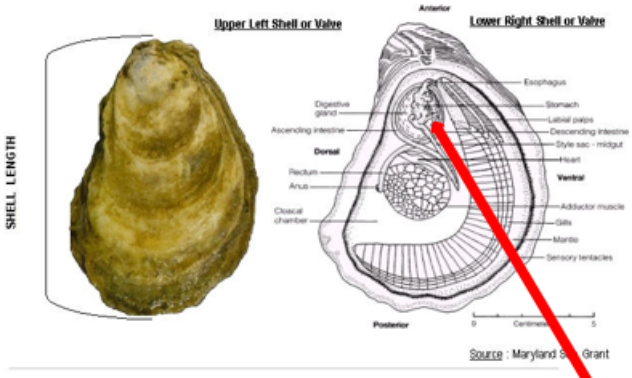
In order to identify etiological viral agents in contaminated molluscan shellfish, it is important to extract and concentrate viruses from shellfish meat because of (a) low viral contamination, (b) naturally occurring inhibitors in shellfish which interfere with the reverse transcription-polymerase chain reaction (RT-PCR), and (c) our inability to culture many human enteric viruses. Since it has been demonstrated that the majority (>70%) of these enteric viruses concentrate in the digestive diverticula of bivalve mollusks (Di Girolamo et al, 1976, Le Guyader et al 2006) this protocol will be applicable for concentration and extraction of enteric viruses from molluscan shellfish where the virus is concentrated in the digestive diverticula such as in oysters, clams, and mussels (Figure C1). In conjunction with the norovirus RT-qPCR assay, this protocol has detected norovirus genogroup I and II (DePaola et al 2010; Woods and Burkhardt 2011; Woods et al., 2016) in molluscan shellfish. Murine norovirus (MNV), ATCC PTA-5935, was used as an extraction control to assess the overall performance of the method.

Single Laboratory Validation Results for the Concentration, Extraction, and Detection of Norovirus and HAV in Molluscan Shellfish

The SLV for the concentration, extraction, and detection of norovirus GI and GII in oysters, mussels, and clams was conducted at GCSL in accordance with a level 2 validation on the Smart Cycler and AB 7500 platforms. For oysters, the extraction control was detected in all replicates and the detection frequency for the medium and low norovirus inoculum was 100% (Table C1 and C2). The norovirus detection frequency for oysters and clams was 100% for the medium inoculum and ranged between 67% and 83% for the low inoculum on the Smart Cycler. The detection frequencies for norovirus in mussels ranged from 78-88% in the medium inoculum and 56-61% for the low. The average C_t values for each matrix showed an estimated ca.1 log difference (3 C_ts) in the norovirus detection for the medium and low level test portions for both the Smart Cycler and AB 7500 platforms (Tables C3, C4, and C5). These results meet the criteria set forth by the FDA Guidelines for the Validation of Analytical Methods for the Detection of Microbial Pathogens in Foods and Feeds, 2nd Ed (2015) for a single laboratory validation.

Figure C1. Anatomy of A) oysters; B) mussels; and C) clams. Only the digestive diverticula is used in concentrating enteric viruses from bivalve shellfish

A



Source: Maryland Sea Grant

B

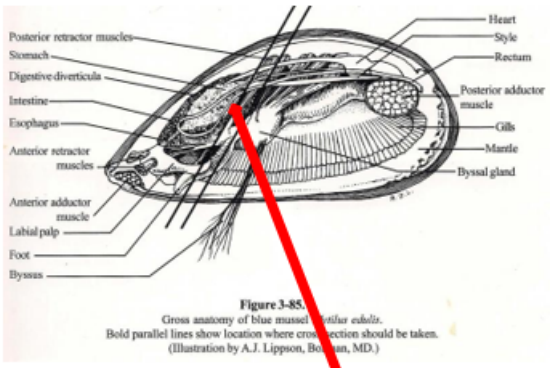
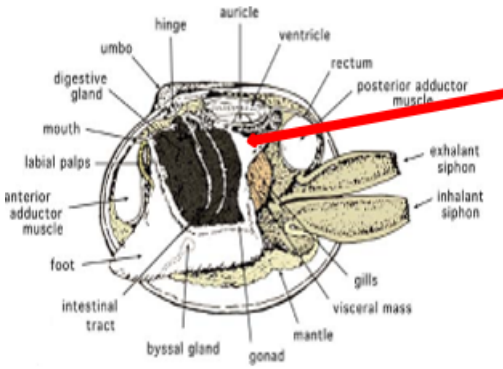


Illustration by A. J. Lippson Bozman, MD

C



Source: FAO Fish and Wildlife Department

Digestive gland used for concentration and extraction of enteric viruses in shellfish.

Table C1. SLV data for norovirus GI and GII in oysters, mussels, and clams on Smart Cycler.

		Medium*	Low~	Uninoculated	MNV EC^
Oysters	GI – Positive samples	18 of 18	12 of 18	0 of 18	18 of 18
Oysters	GI – Percent positive	100	67∞	0	100
Oysters	GII – Positive samples	18 of 18	13 of 18	0 of 18	18 of 18
Oysters	GII – Percent positive	100	72∞	0	100
Mussels	GI – Positive samples	16 of 18	10 of 18	0 of 18	18 of 18
Mussels	GI – Percent positive	88	56∞	0	100
Mussels	GII – Positive samples	14 of 18	10 of 18	0 of 18	18 of 18
Mussels	GII – Percent positive	78	56∞	0	100
Clams	GI – Positive samples	18 of 18	15 of 18	0 of 18	18 of 18
Clams	GI – Percent positive	100	83∞	0	100
Clams	GII – Positive samples	18 of 18	15 of 18	0 of 18	18 of 18
Clams	GII – Percent positive	100	83∞	0	100

***** NoV GI\GII 82.7-175 genomic copies per gram of shellfish
~ NoV GI\GII 8.27-17.5 genomic copies per gram of shellfish
^ MNV extraction control 10^3 PFU/sample
∞ Fractional positives

Table C2. SLV data for norovirus GI and GII in oysters, mussels, and clams on AB 7500.

		Medium*	Low~	Uninoculated	MNV EC^
Oysters	GI – Positive samples	18 of 18	11 of 18	0 of 18	18 of 18
Oysters	GI – Percent positive	100	61∞	0	100
Oysters	GII – Positive samples	16 of 18	12 of 18	0 of 18	18 of 18
Oysters	GII – Percent positive	89	67∞	0	100
Mussels	GI – Positive samples	16 of 18	10 of 18	0 of 18	18 of 18
Mussels	GI – Percent positive	88	56∞	0	100
Mussels	GII – Positive samples	14 of 18	11 of 18	0 of 18	18 of 18
Mussels	GII – Percent positive	78	61∞	0	100
Clams	GI – Positive samples	18 of 18	16 of 18	0 of 18	18 of 18
Clams	GI – Percent positive	100	89∞	0	100
Clams	GII – Positive samples	18 of 18	15 of 18	0 of 18	18 of 18
Clams	GII – Percent positive	100	83∞	0	100

***** NoV GI\GII 82.7-175 genomic copies per gram of shellfish
~ NoV GI\GII 8.27-17.5 genomic copies per gram of shellfish
^ MNV extraction control 10^3 PFU/sample
∞ Fractional positives

Table C3. Mean C_t values for norovirus GI and GII in oysters on the Smart Cycler and AB 7500.

	Smart Cycler – Mean	Smart Cycler – SD	AB 7500 – Mean	AB 7500 – SD	Both Platforms – Mean	Both Platforms – SD
IAC	25.57	2.08	25.24	1.33	25.53	1.84
IAC (2nd aliquot)*	22.73	1.15	23.35	2.37	22.81	1.00
GI Low	39.57	2.30	39.18	1.69	39.38	2.00
GI Medium	37.67	2.01	37.59	2.07	37.63	2.01
GII Low	39.10	3.15	38.66	3.63	38.89	3.33
GII Medium	35.38	2.67	34.73	1.69	35.07	2.26
MNV	33.29	2.77	32.95	2.06	33.12	2.39
MNV (2nd aliquot)*	35.64	3.15	36.88	3.59	36.22	3.38

* New working stocks generated

Table C4. SLV Mean C_t values for norovirus GI and GII in mussels on the Smart Cycler and AB 7500.

	Smart Cycler – Mean	Smart Cycler – SD	AB 7500 – Mean	AB 7500 – SD	Both Platforms – Mean	Both Platforms – SD
IAC	22.71	1.17	23.46	1.09	23.09	1.19
GI Low	40.07	1.68	40.73	2.08	40.54	1.90
GI Medium	37.56	1.92	37.12	1.74	37.34	1.82
GII Low	39.86	2.77	39.71	1.99	39.78	2.33
GII Medium	36.56	2.22	37.34	2.25	36.95	7.20
MNV	36.66	1.85	36.43	2.39	37.61	2.31

Table C5. Mean C_t values for norovirus GI and GII in clams on the Smart Cycler and AB 7500.

	Smart Cycler – Mean	Smart Cycler – SD	AB 7500 – Mean	AB 7500 – SD	Both Platforms – Mean	Both Platforms – SD
IAC	23.84	0.96	24.34	0.51	24.09	0.81
GI Low	38.94	2.86	39.14	1.36	39.05	2.18
GI Medium	35.60	2.63	35.73	2.83	35.69	2.70
GII Low	36.73	2.14	37.70	0.53	37.22	1.61
GII Medium	33.21	3.10	34.30	2.13	33.76	2.68
MNV	33.21	2.82	33.80	2.57	33.52	2.69

Multi-Laboratory Validation Results for the Concentration, Extraction, and Detection of Norovirus and HAV in Molluscan Shellfish

Sample Preparation MLV for Shellfish

Post-harvest processed oysters were purchased from a local market. Each test portion consisted of 12 shucked animals. The oysters' digestive diverticular was removed and aliquoted in 4 gram portions. The number of replicates required for a multi-laboratory validation study is 8 per level inoculated, as stated in the FDA Guidelines for the Validation of Analytical Methods for the Detection of Microbial Pathogens in Foods and Feeds, 2nd Ed (2015). That would have required 4,032 shellfish for the 14 laboratories participating in this study; therefore, we used two spiked levels in triplicate and an uninoculated shellfish in duplicate for a total of 8 samples per laboratory (1,344 total shellfish) for feasibility and equipment limitations. The MLV samples and reagents were prepared and shipped in coolers on dry ice or at refrigerated temperatures, as appropriate, to participating laboratories. Sample analysis began within 24 hours of receipt.

Virus Inocula

The norovirus inocula used for seeding were characterized strains of norovirus GI and norovirus GII. HAV inoculum used for seeding was the vaccine strain (HAV175/18f) propagated in-house utilizing the FrHK cell line. Murine norovirus (MNV) used for seeding was MNV-1 propagated in house using the RAW 264.7 cell line. Three spike levels were used for validation: Low (10 RT-PCR units/g of norovirus GI, 33 RT-PCR units/g of norovirus GII, and 5 PFU/g of HAV), High (100 RT-PCR units/g of norovirus GI, 330 RT-PCR units/g of norovirus GII, and 50 PFU/g HAV) and uninoculated. MNV was inoculated in all samples at 10^3 PFU/g.

Check Samples

Given that the participating laboratories do not routinely perform the shellfish extraction and detection protocol, prior to the MLV, three check samples were sent as a pre-test of the method and analyst performance. The samples consisted of medium, low, and uninoculated shellfish. Each laboratory received the protocols prior to shipping of the check samples to ensure the appropriate supplies and reagents were available. The norovirus protocols from the MLV detection assay were used for the check

samples. The HAV and MNV protocols from BAM chapter 26B were used for the detection of HAV and MNV in shellfish. Reagents for the detection of norovirus, HAV, and MNV detection assays were provided for a total of 60 reactions for the norovirus, MNV, and HAV assays. All other reagent and supplies were of provided by the participating laboratories. Laboratories performed the norovirus and MNV detection assays on the Cepheid Smart Cyclers or the AB 7500s. The HAV detection assay was only performed on the Cepheid Smart Cyclers. Each participating laboratory yielded satisfactory results for extraction and detection of norovirus, HAV, and MNV for the check samples.

Table C6. Laboratory data from detection of norovirus and hepatitis A virus in oysters

Lab	GI Low	GI High	GII Low	GII High	HAV Low	HAV High	Negative Shellfish (Uninoculated)	Extraction Control	RT-qPCR Controls	*Significant Inhibition
Lab #1	3 of 3; 100%	3 of 3; 100%	3 of 3; 100%	3 of 3; 100%	3 of 3; 100%	3 of 3; 100%	2 of 2; 100%	√	√	No
Lab #2	3 of 3; 100%	3 of 3; 100%	3 of 3; 100%	3 of 3; 100%	3 of 3; 100%	3 of 3; 100%	2 of 2; 100%	√	√	No
Lab #3	3 of 3; 100%	3 of 3; 100%	1 of 3 33%$^{\gamma}$	2 of 3 66%$^{\beta}$	3 of 3; 100%	3 of 3; 100%	2 of 2; 100%	√	√	No
Lab #4	3 of 3; 100%	3 of 3; 100%	3 of 3; 100%	3 of 3; 100%	3 of 3; 100%	3of 3; 100%	2 of 2; 100%	√	√	No
Lab #5	3 of 3; 100%	3 of 3; 100%	3 of 3; 100%	3 of 3; 100%	3 of 3; 100%	3 of 3; 100%	1 of 2; 50%+NoV GII$^{\alpha}$	√	√	No
Lab #6	3 of 3; 100%	3 of 3; 100%	3 of 3; 100%	3 of 3; 100%	2 of 3; 66%$^{\gamma}$	3 of 3; 100%	2 of 2; 100%	√	√	No
Lab #7	3 of 3; 100%	3 of 3; 100%	3 of 3; 100%	3 of 3; 100%	3 of 3; 100%	3 of 3; 100%	0 of 2; 0% +NoV GI and GII$^{\alpha}$	√	√	No
Lab #8	3 of 3; 100%	3 of 3; 100%	3 of 3; 100%	3 of 3; 100%	3 of 3; 100%	3 of 3; 100%	0 of 2; 0% +NoV GI and GII, HAV$^{\alpha}$	√	√	No
Lab #9	1 of 3; 33%$^{\gamma}$	3 of 3; 100%	2 of 3; 66%$^{\gamma}$	2 of 3; 66%$^{\beta}$	2 of 3; 66%$^{\gamma}$	3 of 3; 100%	2 of 2; 100%	X$^{\alpha}$	√	No
Lab #10	3 of 3; 100%	3 of 3; 100%	3 of 3; 100%	3 of 3; 100%	3 of 3; 100%	3 of 3; 100%	2 of 2; 100%	√	√	No
Lab #11	Equipment Failure/Incomplete Analysis, No Data Reported									
Lab #12	Analysis Not Completed; No Data Reported									
Lab #13	1 of 3; 33%$^{\gamma}$	3 of 3; 100%	3 of 3; 100%	2 of 3; 66%$^{\beta}$	1 of 3; 33%$^{\gamma}$	1 of 3; 33%$^{\beta}$	2 of 2; 100%	X$^{\alpha}$	√	No
Lab #14	Analysis Not Completed; No Data Reported									

α Denotes invalid lab data/not reported

β Denotes false negatives

γ Denotes fractional positives

* Significant inhibition; internal control C_t for the sample template is ≥4 C_ts for the internal control of the RT-qPCR negative (water) reaction

Table C7. Detection Frequencies of Reporting Laboratories for Norovirus GI, GII, and HAV

Inoculum/gram	**Norovirus GI**	**Norovirus GII**	**HAV**
High^	100%	90%	97%
Low*	88%	90%	88%
Negative (uninoculated)	10%	15%	5%
Real-Time RT-qPCR Controls	**Norovirus GI**	**Norovirus GII**	**HAV**
False negative	0%	0%	0%
False positive	0%	0%	0%

^ Norovirus GI 100 genomic copies/ gram, norovirus GII 330 genomic copies/ gram, HAV 50 PFU/ gram
* Norovirus GI 10 genomic copies/ gram, norovirus GII 33 genomic copies/ gram, HAV 5 PFU/ gram

Conclusion

The concentration, extraction, and detection of enteric viruses in shellfish can be a challenge due to the low levels typically found in naturally contaminated shellfish and the inability to enrich or propagate these viruses. Effective methods for detection of enteric viruses in shellfish matrices are important for outbreak response; therefore, the methods developed should have the ability to detect viruses at low concentrations. Once methods for the detection of enteric viruses in food have been established and validated, laboratories should be able to perform these methods for regulatory analysis. The aim of the study detailed herein was to validate a sensitive and specific method for the concentration, extraction, and detection of enteric viruses from shellfish.

The validation of the norovirus and HAV concentration, extraction, and detection method for molluscan shellfish consisted of a multi-phase approach. No multi-laboratory validated RT-qPCR detection assay for norovirus GI and GII was available at the time. Therefore, the first phase was a MLV of the norovirus RT-qPCR detection assay based on the FDA Guidelines for the Validation of Analytical Methods for the Detection of Microbial Pathogens in Foods and Feeds, 2nd Ed (2015), section 2.3.2 that yielded acceptable results for a "Collaborative Validation Study" (data in Appendix G).

For validation of the concentration and detection assay, the FDA Guidelines for the Validation of Analytical Methods for the Detection of Microbial Pathogens in Foods and Feeds, 2nd Ed (2015), Table 2 – "General Guidelines for the Validation of Qualitative Detection Methods for Microbial Analytes - Unique Isolation and/or Enrichment Challenges" was followed. For the SLV, multiple molluscan shellfish matrices were tested (oysters, clams, and mussels). Detection frequencies for norovirus GI, norovirus GII, and HAV ranged from 78-100% for the high inoculum and 56-89% for the low inoculum.

For the MLV, oyster was selected as a representative matrix for the 13 participating laboratories. The detection frequencies for the 11 labs that submitted results ranged from 88-90% for low shellfish inocula and 90-100% for the high inocula. The level of inhibition is typically minimal with this shellfish protocol, as was the case with this validation. There was little to no inhibition observed (based on IAC C_t values) in the data for the SLV or MLV for the shellfish concentration, extraction, and detection method. Invalid laboratory data in the MLV was due to virus target detection in the negative shellfish or lack of detection of the extraction control in spiked samples.

The results of the validation for the concentration, extraction, and detection of norovirus GI, norovirus GII, and HAV from molluscan shellfish are reproducible and robust. In addition, this shellfish protocol also yields concentrates that can be used for downstream application such as enteric virus characterization and cell culture assays. Our conclusion is that this method can be used for the concentration, extraction, and detection of norovirus and HAV in molluscan shellfish. These assays are ready to be incorporated into the Bacteriological Analytical Manual and ongoing Office of Regulatory Affairs Field Assignments.

Appendix D: Validation Data for the Concentration, Extraction, and Detection of Norovirus Genogroup I, Norovirus Genogroup II, and Hepatitis A Virus from Scallops and Tuna

In the US, hepatitis A virus (HAV) outbreaks associated with the consumption of molluscan shellfish have not occurred since 2005. In addition, there had not been any reports in the US of HAV with scallops as the implicated vehicle. However, in June of 2016, a cluster of HAV illnesses associated with the consumption of imported uncooked bay scallops occurred. The method described here was developed by CFSAN's Gulf Coast Seafood Laboratory as a matrix extension to BAM 26B Detection of Hepatitis A Virus in Foods. This method provides for the concentration, extraction, and detection of enteric viruses from scallop and tuna.

Sample Preparation

Inocula for the matrix extension consisted of cell lysate from the in-house propagation of HAV and murine norovirus (MNV) and chloroform extracted norovirus from clinical samples. QIAamp Viral RNA kit (Qiagen, Carlsbad, CA) was used to extract RNA for HAV and MNV positive controls. RNA transcripts were used for norovirus GI and GII positive controls. All template and real time RT-qPCR reagents were prepared and stored at -20 °C until analysis.

Frozen scallops and tuna loin were purchased from a local retail market. Scallops and tuna, 30 test portions, were thaw, weighted, and placed into Whirl-pak® bags. The test portions were inoculated directly with norovirus GI, GII, and HAV and aged at -20 °C for 72 hrs prior to analysis. All test portions were spiked with an extraction control MNV.

Virus Inoculum

HAV inoculum used for seeding was the vaccine strain (HAV175/18f) propagated in house utilizing FRhK cell line. MNV extraction control used for seeding was MNV-1 propagated in house using RAW 264.7 cell line. Four viral levels were used for the matrix extension for norovirus and HAV; low/LOD, medium, high, and uninoculated. For HAV scallop spikes; high, medium, and low spikes were 10, 1, and 0.1 PFU/g, respectively. For HAV tuna spikes; high, medium, and low spikes were 50, 5, and 0.5 PFU/g, respectively. Due to equipment failure and limited supply of norovirus inoculum, the norovirus scallop spikes were 330 genomic copies/g for the high, 33 for the medium, 3 genomic copies/g for the low inoculum and 0.3 genomic copies for LOD and the norovirus tuna spikes were 1650 genomic copies/g for the high, 165 for the medium, 15 for the low inoculum and 1.5 for the LOD. MNV was inoculated in all samples at 10^3 PFU per sample.

Results

The matrix extension for the concentration, extraction, and detection of norovirus GI, norovirus GII, and hepatitis A virus (HAV) in scallops and finfish meat was conducted at GCSL using the Smart Cycler® and/or AB 7500 platform in accordance with a level 2 (Part-a) validation using Table 1 in the FDA Guidelines for the Validation of Analytical Methods for the Detection of Microbial Pathogens in Foods and Feeds, 2nd Ed (2015). There were two matrices tested, scallops and tuna. Prior to completing this matrix extension, the method was evaluated for emergency use and the LOD for the detection of HAV and norovirus in scallops, tuna, and roe was determined to be 0.1-0.5 plaque forming units per gram (PFU/g) for HAV and 0.3-5 genomic copies per gram (g.c./g) for norovirus. For this protocol, murine norovirus (MNV) was used as the extraction control. MNV was detected in all replicates in both matrices and there was little to no inhibition for the detection of MNV, HAV, and norovirus as indicated in Table D1. For scallops, the average IAC C_t for all three assays was 21.53 with a standard deviation of 0.50. The average IAC for tuna was 21.81 with a standard deviation of 0.67. For the scallops, the average MNV C_t for all three assays was 30.78 with a standard deviation of 0.97, while the average MNV C_t in tuna for all three assays was 32.33 with a standard deviation of 1.95, Table 2. Norovirus GI, GII, and HAV were detected in all replicates for high and medium inoculum for scallops and tuna (Table D3 and D4). The detection frequencies for fractional positives, represented by the low and LOD inoculum, ranged from 20 to 80% for scallops and 20 to 100% for tuna.

Table D1. Average internal control C_t values for MNV, HAV, and norovirus in scallops and tuna

Sample #	MNV (Scallops)	HAV (Scallops)	Norovirus (Scallops)	MNV (Tuna)	HAV (Tuna)	Norovirus (Tuna)
1	21.50	21.33	21.12	21.47	20.86	22.45
2	21.40	21.22	21.34	21.22	20.86	22.79
3	21.60	21.29	21.17	21.38	20.91	22.30
4	21.69	21.39	21.36	21.31	21.03	22.39
5	21.37	21.30	20.90	21.55	21.00	22.47
6	21.63	21.40	21.07	21.48	20.91	22.50
7	21.48	21.32	21.33	21.56	20.96	22.48
8	21.17	21.33	21.25	21.28	20.96	22.48
9	21.72	21.63	21.49	21.71	21.18	22.46
10	21.38	21.40	21.17	21.61	21.01	22.32
11	21.47	21.33	21.30	21.79	21.38	22.36
12	21.38	21.33	21.14	21.43	21.16	22.40
13	21.66	21.46	21.44	21.83	21.49	22.37
14	21.63	21.75	23.71	21.53	21.38	22.42
15	21.56	21.43	21.21	21.25	20.95	22.60
16	21.35	21.46	21.28	21.10	21.10	22.66
17	21.59	21.35	21.30	22.03	21.62	23.05
18	21.65	21.87	21.53	21.31	20.90	22.74
19	23.33	23.36	23.58	21.95	21.44	23.06
20	21.67	21.74	21.73	21.76	21.21	23.00
21	22.15	22.05	21.82	21.82	21.25	23.06
22	21.99	21.85	21.58	21.39	20.92	22.89
23	21.74	21.69	21.37	22.01	21.39	23.07
24	22.15	22.09	21.39	21.59	20.86	22.64
25	21.25	21.28	21.20	21.67	21.12	22.71
26	21.21	21.37	21.29	21.45	21.25	22.60
27	21.17	21.34	21.15	21.96	21.57	22.64
28	21.26	21.70	20.99	22.02	21.41	22.97
29	21.44	21.13	21.04	22.18	21.83	23.07
30	21.27	21.19	20.99	21.67	21.26	22.68
Positive control	21.30	21.32	21.30	21.36	20.80	22.35
Negative control	21.45	21.63	21.42	21.55	21.17	22.57

Table D2. Average MNV C_t values for scallop and tuna samples

Sample #	MNV C_t Averages (Scallops)	MNV C_t Averages (Tuna)
1	30.45	33.51
2	29.92	33.14
3	29.61	34.88
4	29.56	37.18
5	29.91	34.97
6	29.62	36.37
7	30.63	33.63
8	30.78	33.51
9	29.20	33.06
10	31.17	33.91
11	30.60	31.70
12	32.34	32.74
13	31.30	33.76
14	31.10	33.20
15	31.74	31.73
16	32.09	31.10
17	30.47	30.53
18	29.23	31.50
19	29.23	30.04
20	32.27	29.99
21	30.34	30.19
22	31.27	30.42
23	31.44	29.64
24	30.65	31.01
25	32.84	31.23
26	31.83	32.65
27	31.13	32.13
28	31.27	30.14
29	30.78	29.89
30	30.70	32.09

Table D3. Norovirus GI, norovirus GII, and HAV detection in scallops

	Medium[a] 33 g/c	**Low[b] 3 g/c**	**LOD[c] 0.3 g/c**	**Uninoculated 0 g/c**	**MNV EC[e] 10^2 PFU/g[f]**
norovirus GI – Positive Samples	5 of 5	7 of 15[d]	1 of 5[d]	0 of 5	30 of 30
norovirus GI – Percent Positive	100	47	20	0	100
	High[g] 330 g/c	**Medium 33 g/c**	**Low 3 g/c**	**Uninoculated 0 g/c**	**MNV EC[e] 10^2 PFU/g**
norovirus GII – Positive Samples	5 of 5	15 of 15	4 of 5[d]	0 of 5	30 of 30
norovirus GII – Percent Positive	100	100	80	0	100
	Medium 10 PFU/g	**Low 1 PFU/g**	**LOD 0.1 PFU/g**	**Uninoculated 0 g/c**	**MNV EC[e] 10^2 PFU/g**
HAV – Positive Samples	5 of 5	12 of 15[d]	1 of 5[d]	0 of 5	30 of 30
HAV – Percent Positive	100	80	20	0	100

[a] NoV GI 33 genomic copies per gram scallop
[b] NoV GI\GII 3 genomic copies per gram scallop
[c] LOD Limit of Detection
[d] Fractional positives
[e] MNV extraction control
[f] Plaque Forming Units per gam
[g] NoV GII 330 genomic copies per gram scallop

Table D4. Norovirus GI, norovirus GII, and HAV detection in tuna

	Medium[a] 165 g/c	**Low[b] 15 g/c**	**Low/LOD[c] 1.5 g/c**	**Uninoculated 0 g/c**	**MNV EC[e] 10^2 PFU/g[f]**
norovirus GI – Positive Samples	5 of 5	4 of 15[d]	3 of 5[d]	0 of 5	30 of 30
norovirus GI – Percent Positive	100	27	60	0	100
	High[g] 1650 g/c	**Medium 165 g/c**	**Low 15 g/c**	**Uninoculated 0 g/c**	**MNV EC[e] 10^2 PFU/g**
norovirus GII – Positive Samples	5 of 5	15 of 15	4 of 5[d]	0 of 5	30 of 30
norovirus GII – Percent Positive	100	100	80	0	100
	Medium 50 PFU/g	**Low 5 PFU/g**	**Low/LOD 0.5 PFU/g**	**Uninoculated 0 g/c**	**MNV EC[e] 10^2 PFU/g**
HAV – Positive Samples	5 of 5	15 of 15	5 of 5	0 of 5	30 of 30
HAV – Percent Positive	100	100	100	0	100

[a] NoV GI 165 genomic copies per gram tuna
[b] NoV GI\GII 15 genomic copies per gram tuna
[c] LOD Limit of Detection
[d] Fractional positives
[e] MNV extraction control
[f] Plaque Forming Units per gam
[g] NoV GII 1650 genomic copies per gram tuna

Conclusion

Prior to the 2016 hepatitis A virus (HAV) scallop outbreak, there was no FDA method for the concentration, extraction, and detection of enteric viruses in scallops or tuna. BAM Chapter 26B contained a protocol for the concentration, extraction, and detection of HAV that involves a direct elution of enteric viruses from the surface of green onion. This method was applied to scallops and tuna using a slight modification of an amino acid buffer and was proven successful in concentration, extraction, and detection of HAV from scallops and tuna. In addition to the results provided in this document, this protocol has been used to analyze over 50 samples related to the 2016 scallop outbreak and associated import bulletin in “emergency use” status.

The extraction control was detected in all the spiked replicates and there was little to no inhibition in any of the sample replicates. The detection frequency of the method was 100% for both the high and medium inocula and ranged from 20-100% for the low inocula. The data interpretation of the detection assay portion of this method includes recommendations on the next steps to take should the sample exhibit inhibition (BAM Chapter 26, Sections B2 and B4). The results show the assay is sensitive, reproducible, and robust and has established the "Fitness of Purpose" for concentration, extraction, and detection of norovirus GI, norovirus GII, and HAV from scallops and tuna.

Our conclusion is that this matrix extension can be used for the concentration, extraction, and detection of norovirus and HAV in scallops and tuna. This protocol is ready to be incorporated into the Bacteriological Analytical Manual and ongoing Office of Regulatory Affairs Field Assignments.

Appendix E: Data Analysis and Supplemental Material for the Murine Norovirus Detection Assay

The murine norovirus (MNV) RT-qPCR detection assay is used to assess the recovery of murine norovirus from spiked samples and to determine if the extraction was performed correctly. Valid norovirus and/or HAV sample results are contingent upon the successful detection of the extraction control from the sample being tested. Figures E1 and E2 demonstrate typical linear and log amplification curves for the RT-qPCR assays. Figures E3 and E4 demonstrate false positives or non-linear amplification. The MNV assay utilizes IAC primers and probe that are multiplexed (simultaneously amplified) with MNV primers and probe for each RNA sample. All primers and probes should be hydrated to a concentration of 100 µM using primer TE prior to making primer and probe mixes.

Data Analysis for the Murine Norovirus Detection Assay

Figure E1. Linear amplification plot of positive virus sample. This is a typical representation of a positive sample.

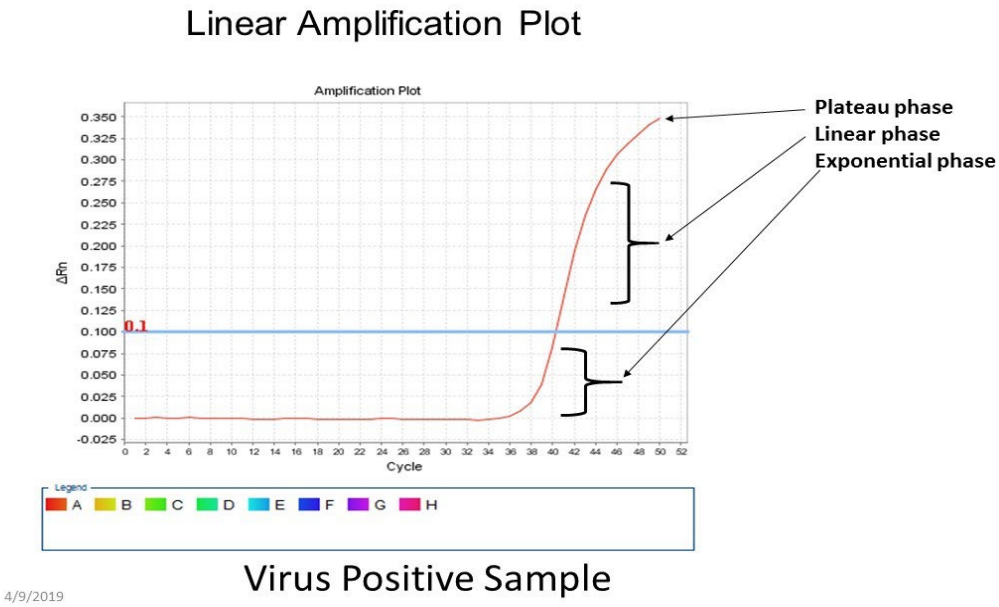


Figure E2. Log amplification plot of positive virus sample. This is a typical representation of a positive sample.

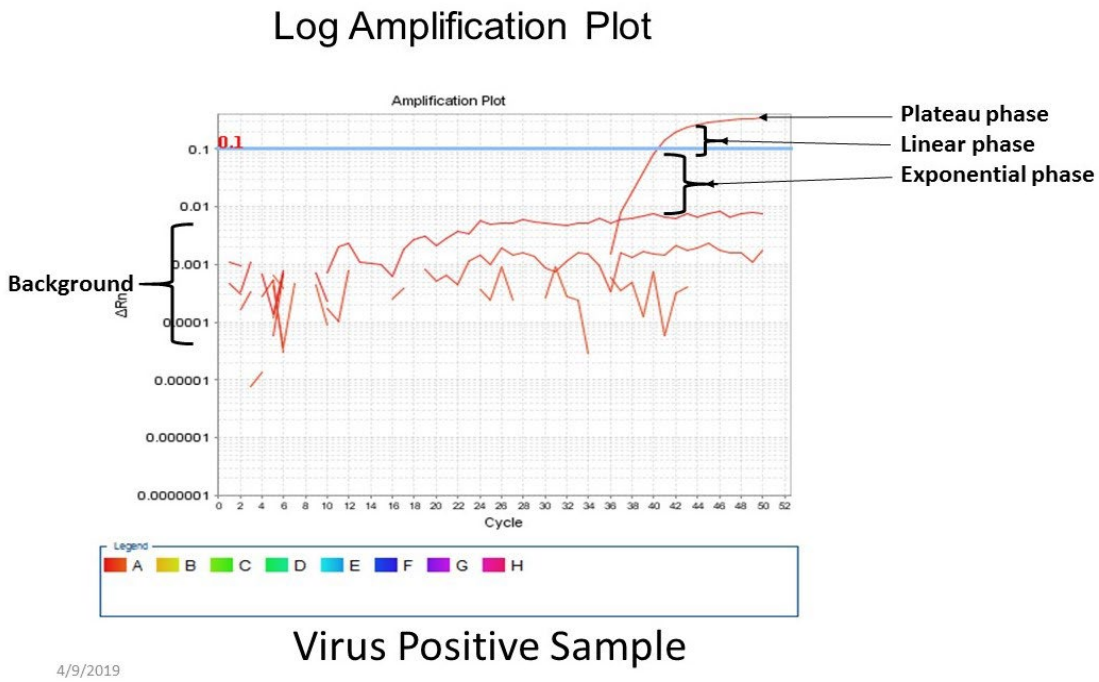


Figure E3. Linear amplification plot of false positive virus sample.

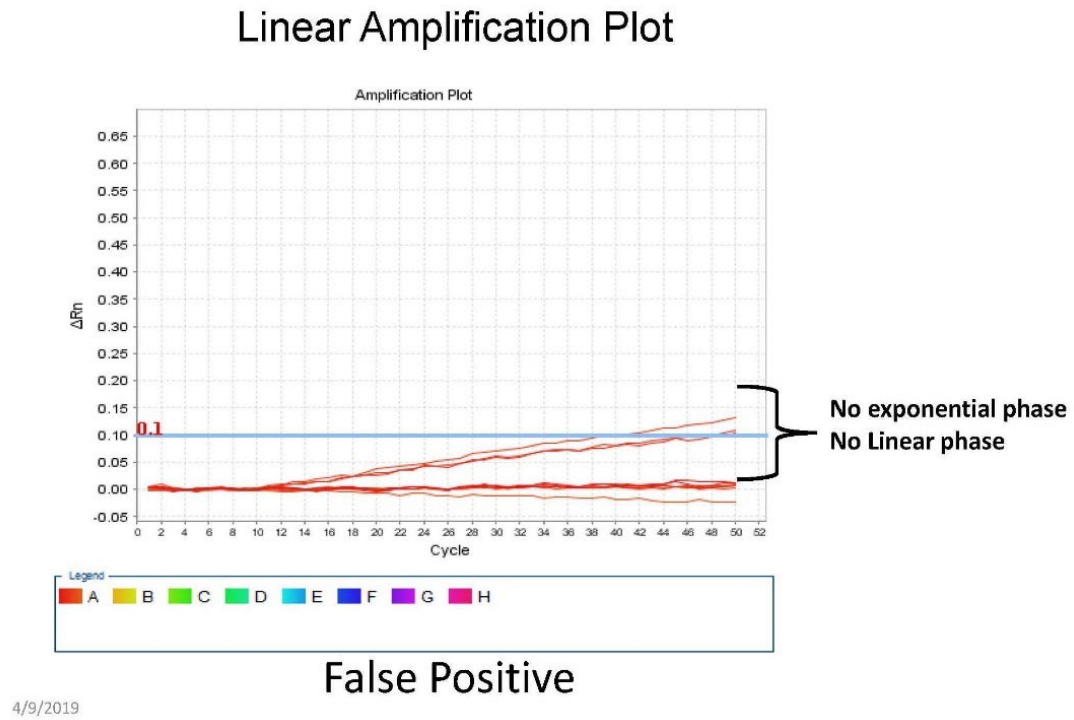
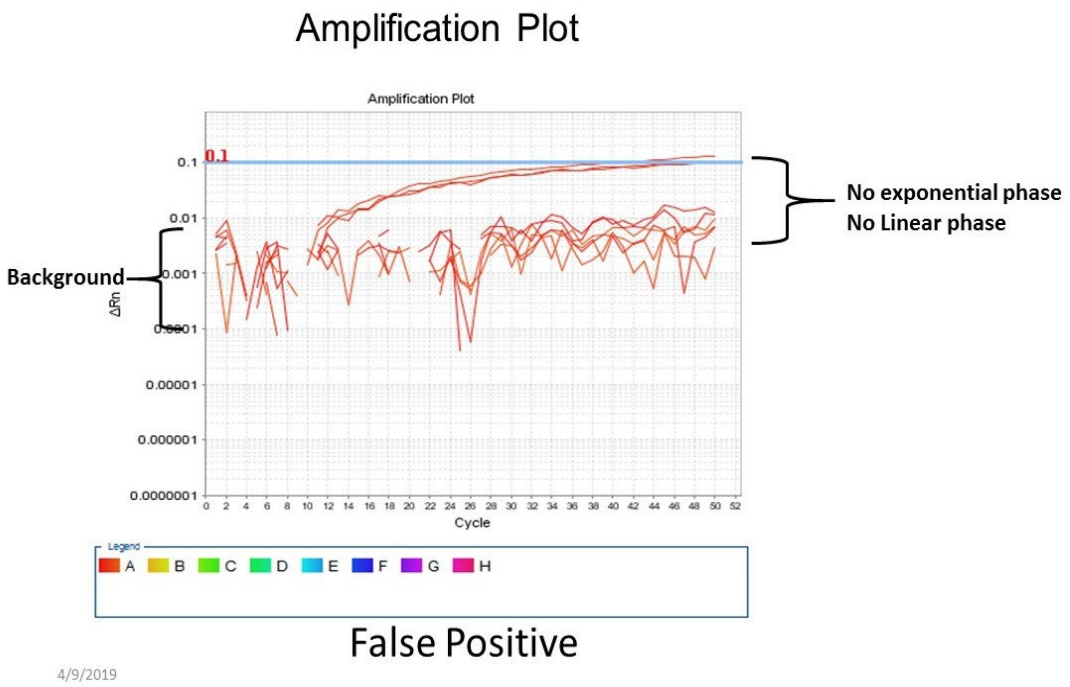


Figure E4. Log amplification plot of false positive virus sample.



Supplemental Material for the Murine Norovirus Detection Assay

Table E1. Master Mix components for RT-qPCR assays for AB 7500

Reagent	**Volume per 25 µl for 1 reaction**	**Volume for 75 reactions**
Buffer Mix	15.55 µl	1875 µl
Primer Mix 2-plex	2 µl	150 µl
Probe Mix	1 µl	75 µl
Enzyme Mix	1.25 µl	93.75 µl
FAM dye	2 µl	150 µl
Internal Control RNA*	0.2 µl	15 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.

Table E2. AB7500 HAV, MNV, and Norovirus Buffer Mix Setup*

Reagents	**Volume (50 mM MgCl)**	**Volume (25 mM MgCl)**
DNase/RNase free H_2O	1760 µl	1610 µl
5X Buffer	1000 µl	1000 µl
MgCl	150 µl	300 µl
dNTPs	200 µl	200 µl

* Made with components from Qiagen One-Step RTqPCR kit and PCR grade water

Table E3. Enzyme Mix Setup

Component	**Volume**
One-Step Qiagen enzyme	~200 µL
Superase-IN (10000U)	50 µL

Table E4. MNV Primer Mix Setup*

Primers/H_2O	**Volume**
MNVF	12.5 µl
MNVR	12.5 µl
IC46	4.69 µl
IC194	4.69 µl
DNase/RNase water	465.62 µl

* Made with 100 µM primers stocks and PCR grade water

Table E5. MNV Probe Mix

Probe/H_2O	**Volume**
MNVP	12.5 µl
ICP	18.75 µl
DNase/RNase water	468.75 µl

* Made with 100 µM probe stocks and PCR grade water

Appendix F: Validation Data, Data Analysis, and Supplemental Material for the Hepatitis A Virus Detection Assay and Control Exclusion Assay

Single Laboratory Validation of HAV RT-qPCR Detection Assay

The real time RT-qPCR assay for HAV was validated for use on the Cepheid® SmartCycler in multiple phases. For the SLV of the HAV detection assay, three HAV strains were used to establish inclusivity (Table F1). Eleven enteric viruses and five pathogenic enteric bacterial species were used to establish assay exclusivity (Table F2). HAV-positive and HAV-negative human sera samples from CDC were tested using the HAV detection assay and results demonstrated 100% accuracy in detection (Table F3). The amplification efficiency ranged between 97% and 103% (Table F4). There was no inhibition in the assay with the addition of the internal amplification control (IAC) and competitive RNA poliovirus (Table F5). The dynamic range of the assay was 7 logs (Figure F1) and the limit of quantification (LOQ) and limit of detection (LOD) were 0.11 and 0.001 PFU/reaction, respectively (Table F6).

RNA Template Controls and Clinical Specimens

Viral Nucleic Acid Templates (for inclusivity/exclusivity testing)

Template RNA was isolated from stock suspensions and diluted for inclusive and exclusive, viruses (Tables F1 and F2) and stool samples using the QIAamp Viral RNA Mini Kit (Qiagen) protocol for cell culture. The RNA was eluted from the spin columns with 60 µl AVE elution buffer (provided in kit) and stored at -80 °C until used.

RNA Template for Competitive RNA Testing

Poliovirus RNA was isolated from stock suspensions, diluted, and added to the HAV RT-qPCR multiplex assay to determine if the presence of additional enteric viral RNA would be competitive with detection of HAV viral RNA. The poliovirus RNA was extracted using the QIAamp Viral RNA Mini Kit (Qiagen) protocol for cell culture. The RNA was eluted from the spin columns with 60 µl AVE elution buffer (provided in kit) and stored at -80 °C until used.

Bacterial Templates (for exclusivity testing)

DNA templates were prepared by transferring 1 ml of overnight Tryptic Soy Broth culture to a microcentrifuge tube and centrifuged at 12,000 × *g* for 3 min. The supernatant was removed and the pellet completely resuspend in 1 ml 0.85% NaCl. The tube was centrifuged 12,000 × *g* for 3 min. The supernatant was removed and the pellet was completely re-suspended in 1 ml sterile water. The tube was place in a water bath or heat block and maintained at 100 °C for 10 min. Following boiling the tube was then centrifuged 12,000 × *g* for 1 min, and the supernatant was removed and transferred to a new microcentrifuge tube. This bacterial extracted was frozen at -20 °C and served as the appropriate control.

Table F1. Inclusivity of the HAV detection assay

Strain	Source ATCC	HAV Average Ct*	SD	IAC Average Ct	SD	Frequency
HM175/18f (sub-genotype 1B)	VR-1402	29.08	0.208	20.88	0.22	6 of 6
PA21 (sub-genotype IIIA)	VR-1357	23.24	0.261	20.85	0.33	6 of 6
PA21 (sub-genotype IIIA)	VR-2281	20.07	0.352	21.221	1.08	6 of 6

*Average Ct value of 6 replicates.

Table F2. Exclusivity testing of the HAV detection assay

Organism	Source	Results	Frequency of Result
Poliovirus	ATCC VR-193	Negative	6 of 6
Astrovirus	HuAst1	Negative	6 of 6
San Miguel Sea Lion virus serogroup 17	Dr. Alvin Smith, Univ. OR, Corvallis	Negative	6 of 6
Rotavirus	ATCC VR 2018	Negative	6 of 6
Adenovirus	ATCC VR-1083	Negative	6 of 6
Feline Calicivirus	ATCC VR-2057	Negative	6 of 6
Human Paraechovirus	ATCC VR-1063	Negative	6 of 6
Echovirus 1	ATCC VR-1038	Negative	6 of 6
Coxsackievirus	ATCC VR-1007	Negative	6 of 6
Norwalk Virus GI	Human Stool	Negative	6 of 6
Norovirus GII	Human Stool	Negative	6 of 6
Escherichia coli	ATCC 25922	Negative	6 of 6
Salmonella enterica	ATCC 9700	Negative	6 of 6
Shigella sonnei	ATCC 9290	Negative	6 of 6
Vibrio cholerae	ATCC 14035	Negative	6 of 6
Listeria monocytogenes	ATCC 7646	Negative	6 of 6

Table F3. Specificity testing of HAV detection assay with human serum samples

Sample Number	HAV Detection	C_t Value*	IAC C_t Value
17000	Positive	34.65	23.63
14000	Positive	32.23	23.16
13516	Positive	25.67	23.14
12010	Positive	39.86	22.74
12009	Positive	27.18	23.42
17500	Positive	37.49	23.53
12144	Positive	28.74	23.03
16000	Positive	36.25	23.33
12121	Positive	23.91	22.82
12113	Positive	28.66	23.65
12101	Positive	23.27	23.15
12112	Positive	29.21	23.58
13518	Positive	32.42	23.32
12319	Positive	29.73	23.61
12399	Positive	29.75	23.11
12320	Positive	28.25	23.18
12312	Positive	30.68	23.19
12323	Positive	28.58	23.33
12322	Positive	26.80	23.34
12305	Positive	25.12	22.87
12330	Positive	24.25	23.10
12385	Positive	28.26	23.19
12316	Positive	26.09	23.21
12346	Positive	33.88	23.18
12363	Positive	28.09	23.24

Sample Number	HAV Detection	C_t Value*	IAC C_t Value
12325	Positive	22.38	23.22
12364	Positive	32.32	23.21
12359	Positive	31.70	23.12
12326	Positive	28.10	23.60
12302	Positive	29.08	23.58
12313	Positive	33.28	23.28
12352	Positive	33.74	23.40
12303	Positive	29.06	23.32
12304	Positive	26.83	23.55
12306	Positive	28.95	23.53
12329	Positive	29.44	22.75
12330	Positive	25.12	23.35
12307	Positive	31.42	23.29
12331	Positive	31.35	23.15
12345	Positive	28.54	23.12
12003	Negative**	-	23.41
12013	Negative**	-	23.34
19300	Negative**	-	23.23
13517	Negative**	-	23.28

*Serum samples tested in a single reaction due to limited amount of serum provided
**Negative for HAV based on CDC's data

Table F4. HAV RT-qPCR Intra-Assay Variability: 3 - 100-fold dilutions of HAV RNA

	Trial 1			Trial 2			Trial 3			Trial 4			Trial 5		
Ct*	**High**	**Med**	**Low**	**High**	**Med**	**Low**	**High**	**Med**	**Low**	**High**	**Med**	**Low**	**High**	**Med**	**Low**
Mean	21.09	27.85	34.37	28.24	34.82	21.38	27.92	34.70	34.73	21.26	28.24	34.82	21.38	27.92	34.70
SD	0.182	0.320	0.243	0.250	0.599	0.249	0.338	0.523	0.347	0.350	0.250	0.599	0.249	0.338	0.523
SE	0.061	0.103	0.061	0.083	0.200	0.083	0.113	0.198	0.116	0.117	0.083	0.200	0.083	0.113	0.198
Amplification Efficiency	100%			103%			97%			97%			100%		
r^2	0.998			0.998			0.997			0.995			0.996		
Internal Control Impact	No Significant Difference (p= 0.113)			No Significant Difference (p= 0.415)			No Significant Difference (p= 0.183)			No Significant Difference (p= 0.311)			No Significant Difference (p= 0.939)		

*From 9 replicates
Mean Efficiency: 100%; $r^2 = 0.997$

Intra-Assay Reproducibility: Five samples of varying low (C_t 33-36) medium (C_t 27-28) and high (C_t 21-22) concentrations were tested in 9 reactions on the same run. The results show excellent reproducibility.

Inter-Assay Reproducibility: The C_t value of the daily positive control was analyzed over a period of 3 days on two different, calibrated, Smart Cycler blocks. The results show consistent reproducibility over time on the same sample.

Table F5. Detection of HAV and IAC in the presence of competitive RNA

	HAV Detection				IAC Detection			
	No Competitor		**With Polio at 4×10^4 pfu/rxn**		**No Competitor**		**With Polio at 4×10^4 pfu/rxn**	
	Average C_t*	**SD**	**Average C_t**	**SD**	**Average C_t***	**SD**	**Average C_t**	**SD**
HAV 2×10^3 pfu/rxn	27.06	0.164	26.97	0.207	22.36	0.126	22.28	0.094
HAV 20 pfu/rxn	32.90	0.326	33.42	0.408	22.18	0.264	22.31	0.278
HAV 0.2 pfu/rxn	41.03	0.768	40.72	1.440	22.34	0.129	22.41	0.193

*Average of 6 replicates.

Figure F1. Dynamic Range of Assay: The assay has a dynamic range of 7 logs and a mean efficiency of 99.4%

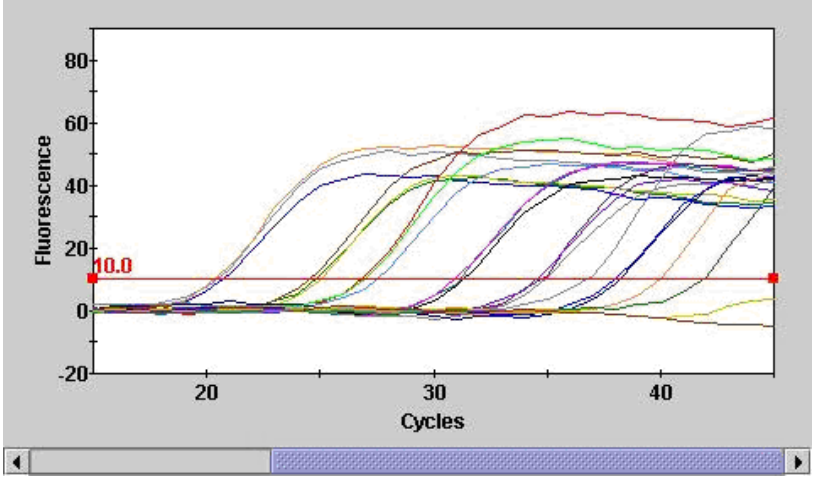


Figure F2. Standard Curve

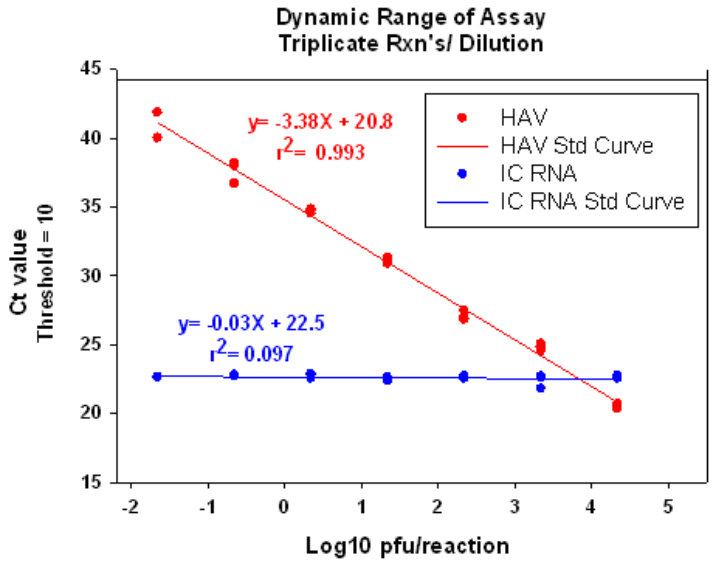


Table F6. Limit of Detection (LOD)/Limit of Quantitation (LOQ) using HAV HM175/18f

	Average C_t	SD*	Pos/Total
0.11 PFU	41.13	0.55	10/10
0.01 PFU	42.44	0.82	7/10
0.001 PFU	43.48	1.04	8/10

* Standard deviation of positive samples only.
LOQ = 0.11 PFU/rxn
LOD = 0.001 PFU/rxn

Multi-Laboratory Validation of HAV RT-qPCR Detection Assay

The MLV of the HAV detection assay consisted of four stages conducted by eight participating FDA laboratories. Each of the four stages included a minimum of four laboratories. Microorganisms tested for inclusivity and exclusivity included three strains of HAV, four enteric viruses, and enteric bacteria (Table F7). The inter-laboratory repeatability results demonstrated outliers for two laboratories but these results should be considered analyst error and not a function of the analytical tests performed (Table F17). Overall, results for the MLV demonstrated an accuracy of 99% with a 1% false positive and false negative rate (Table F18). These accuracy rates are within the acceptable limits for Nucleic Acid Technology (NAT) assays.

Sample Preparation

Template for the detection assay consisted of extracted RNA using QIAmp Viral RNA kits (Qiagen, Carlsbad, CA). For the four stages of the MLV, each stage was completed 1 to 2 months apart. Template and PCR reagents were shipped overnight on dry ice and stored at -20 °C until analysis.

Table F7. Microorganisms Tested in Multi-Laboratory Validation

Strain
HAV HM 175/18f
HAV PA 21
HAV HAS15
Poliovirus
Astrovirus
San Miguel Sea Lion virus serogroup 17
Norovirus GII
Salmonella enterica

6 replicates/ organism were generally tested but, due to laboratory error, only 3 replicates were reported in certain instances.

Table F8. Stage #1 Results

Detection of HAV via RT-qPCR				
Organism/ Strain	**Lab #1**	**Lab #5**	**Lab #6**	**Lab #7**
HAV HM- 175	3 of 3	6 of 6	6 of 6	6 of 6
HAV PA 21	3 of 3	6 of 6	6 of 6	6 of 6
HAV HAS15	3 of 3	6 of 6	6 of 6	6 of 6
Poliovirus	0 of 3	0 of 6	0 of 6	0 of 6
Astrovirus	0 of 3	0 of 6	0 of 6	0 of 6
NoV; SMSV-17	0 of 3	0 of 6	0 of 6	0 of 6
HuNoV GII	0 of 3	0 of 6	0 of 6	0 of 6
Salmonella	0 of 3	0 of 6	0 of 6	0 of 6

HAV: 63 of 63 reactions positive; 100% accuracy

Non-HAV: 105 of 105 reactions negative; 100% accuracy

Table F9. Stage #2 Results

Detection of HAV via RT-qPCR					
Organism/ Strain	**Lab #1**	**Lab #2**	**Lab #3**	**Lab #4**	**Lab #5**
HAV HM-175	6 of 6	6 of 6	6 of 6	6 of 6	6 of 6
HAV PA 21	6 of 6	6 of 6	6 of 6	6 of 6	6 of 6
HAV HAS15	5 of 6	6 of 6	6 of 6	6 of 6	6 of 6
Poliovirus	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6
Astrovirus	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6
NoV; SMSV-17	0 of 6	2 of 6	0 of 6	0 of 6	0 of 6
HuNoV GII	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6
Salmonella	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6

HAV: 89 of 90 reactions positive; 99% accuracy

Non-HAV: 178 of 180 reactions negative; 99% accuracy

Table F10. Stage #3 Results

Detection of HAV via RT-qPCR				
Organism/ Strain	**Lab #1**	**Lab #4**	**Lab #6**	**Lab #7**
HAV HM- 175	6 of 6	6 of 6	6 of 6	6 of 6
HAV PA 21	6 of 6	6 of 6	6 of 6	6 of 6
HAV HAS15	6 of 6	6 of 6	6 of 6	6 of 6
Poliovirus	0 of 6	0 of 6	0 of 6	0 of 6
Astrovirus	1 of 6	0 of 6	0 of 6	0 of 6
NoV; SMSV-17	0 of 6	0 of 6	0 of 6	0 of 6
HuNoV GII	0 of 6	0 of 6	0 of 6	0 of 6
Salmonella	0 of 6	0 of 6	0 of 6	0 of 6

HAV: 72 of 72 reactions positive; 100% accuracy

Non-HAV: 179 of 180 reactions negative; 99% accuracy

Table F11. Stage #4 Results

Detection of HAV via RT-qPCR					
Organism/Strain	**Lab #1**	**Lab #2**	**Lab #5**	**Lab #7**	**Lab #8**
HAV HM- 175	6 of 6	6 of 6	6 of 6	6 of 6	6 of 6
HAV PA 21	6 of 6	6 of 6	6 of 6	6 of 6	6 of 6
HAV HAS15	6 of 6	6 of 6	6 of 6	6 of 6	6 of 6
Poliovirus	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6
Astrovirus	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6
NoV; SMSV-17	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6
HuNov GII	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6
Salmonella	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6

HAV: 89 of 90 reactions positive; 99% accuracy

Non-HAV: 149 of 150 reactions negative; 99% accuracy

Table F12. Inter-Laboratory Repeatability - Stage #1

HAV Strain	Lab #1			Lab #2			Lab #3			Lab #4			Lab #5		
	Mean*	SD	SE	Mean	SD	SE	Mean	SD	SE	Mean	SD	SE	Mean	SD	SE
HM-175	25.1	0.21	0.09	27.9**	0.77	0.31	25.4	0.35	0.14	25.3	0.31	0.13	25.2	0.16	0.06
PA 21	24.5	0.20	0.08	27.7**	0.18	0.07	24.7	0.68	0.28	24.2	0.19	0.08	24.3	0.36	0.15
HAS-15	26.3	0.19	0.08	26.3	0.40	0.16	27.2	1.21	0.50	26.6	0.51	0.21	26.2	0.21	0.09

HAV HM-175: 1.3×10^3 pfu/rxn; HAV PA 21: 7.2×10 pfu/rxn; HAV HAS-15: 1.1×10 pfu/rxn
* Means, SD (standard deviations), and SE (standard errors) of 6 replicates
** Results from Lab #2 are significantly different than results from the other 4 laboratories

Table F14. Inter-Laboratory Repeatability - Stage #3

HAV Strain	Lab #1			Lab #4			Lab #6			Lab #7		
	Mean*	SD	SE	Mean	SD	SE	Mean	SD	SE	Mean	SD	SE
HM-175	30.3	0.21	0.09	29.9	0.46	0.19	29.4	0.18	0.07	28.7^	0.44	0.18
PA 21	30.5	0.44	0.18	29.8	0.22	0.09	30.0	0.17	0.07	29.3	0.24	0.10
HAS-15	30.6	0.66	0.27	30.8	0.69	0.07	30.1	0.17	0.07	29.7	0.42	0.17

HAV HM 175 - 1.3×10^1 pfu/rxn; HAV PA21 - 7.3×10^1 pfu/rxn; HAV HAS-15 - 1.1×10^2 pfu/rxn
* Means, SD (standard deviations), and SE (standard errors) of 6 replicates
^ Results from Lab #7 are significantly different than results from the other 4 laboratories

Table F15. Inter-Laboratory Repeatability - Stage #4

HAV Strain	Lab #1			Lab #2			Lab #5			Lab #7			Lab #8		
	Mean*	SD	SE	Mean	SD	SE	Mean	SD	SE	Mean	SD	SE	Mean	SD	SE
HM-175	28.2	0.59	0.24	27.7	0.27	0.11	28.0	0.42	0.17	24.5^	1.39	.055	28.4	0.34	0.14
PA 21	28.7	0.33	0.14	28.7	0.44	0.18	27.99^{+}	0.30	0.12	28.6	0.23	0.09	28.7	0.18	0.08
HAS-15	28.6	0.23	0.10	32.2[a]	1.02	0.42	28.4	0.22	0.09	28.4	0.18	0.07	28.9	0.23	0.10

HAV HM-175: 1.3×10^2 pfu/rxn; HAV PA 21: 9.5×10^2 pfu/rxn; HAV HAS-15: 2.2×10^3 pfu/rxn
* Means, SD (standard deviations), and SE (standard errors) of 6 replicates
^ Results from Lab #7 are significantly different than results from the other 4 laboratories
$^{+}$ Results from Lab #5 are significantly different than results from the other 4 laboratories
[a] Results from Lab #2 are significantly different than results from the other 4 laboratories

Table F16. Trial Inter-Laboratory Repeatability – Summary

HAV Strain	Trial #1			Trial #2			Trial #3			Trial #4		
	Mean*	SD	SE	Mean	SD	SE	Mean	SD	SE	Mean	SD	SE
HM-175	22.0	0.28	0.06	25.8	1.16	0.21	29.6	0.66	0.13	27.4	1.63	0.30
PA 21	21.3	0.34	0.08	25.1	1.38	0.25	29.9	0.51	0.10	28.5	0.40	0.07
HAS-15	23.9	0.31	0.07	26.5	0.72	0.13	30.3	0.65	0.13	28.6	2.44	0.45

* Means, SD (standard deviations), and SE (standard errors) of 6 replicates per laboratory; 24 replicates for Trials # 1 and #3; 30 replicates for Trials #2 and #4

Table F17. Summary of Detection: Inclusivity and Exclusivity 8 labs over 4 trials

Organism / Strain	Accuracy	False Negatives	False Positives
HAV HM-175	100%	0%	-
HAV PA 21	100%	0%	-
HAV HAS-15	98%	2%	-
Poliovirus	100%	-	2%
Astrovirus	99%	-	1%
Norovirus; SMSV-17	98%	-	2%
Human Norovirus GII	100%	-	0%
Salmonella	100%	-	0%
All HAV Strains	**99%**	**1%**	
All Other Organisms/Strains	**99%**	**-**	**1%**

Validation of Platform Extension of the HAV RT-qPCR Detection Assay to the AB 7500

The multi-laboratory validated method for the detection of hepatitis A virus (HAV) by RT-qPCR was conducted on the Smart Cycler platform. In order to expand the utility of the assay, a platform extension of the FDA BAM 26B HAV RT-qPCR assay was performed on the AB 7500. For the HAV assay on the AB 7500, the cycling conditions, master mix components, and template concentration is identical to the validated assay in the FDA BAM 26B. The AB 7500 platform requires a reference dye to normalize the data. Instead of the widely used ROX dye, FAM was added to the master mix as the passive reference dye. The assay was examined for sensitivity, reproducibility, robustness, LOD, and LOQ (Tables F18-19). In order to evaluate stability of this assay using 50 cycles, a 96-well plate with no template control (PCR grade water) and a 96-well plate of negative finfish matrix was evaluated on AB 7500 platform (Tables F20 and F21). Assessment of the assay in 3 for medium and low inoculum 2 uninoculated for frozen strawberries was also completed (Table F22).

Sample Preparation

RNA Template Controls and Spikes

HAV RNA was extracted from stock suspensions of the laboratory strain (HAV175/18f) using the QIAamp Viral RNA Mini Kit (Qiagen) protocol for cell culture. The RNA was eluted from the spin columns with 60 µl AVE elution buffer (provided in kit) and stored at -80 ºC until used. Extracted HAV RNA was used template for the RT-qPCR assays. PCR grade water was used for the no template control plate.

Virus Inocula for Berry Matrix

HAV inoculum used for seeding was the vaccine strain (HAV175/18f) propagated in-house utilizing the FrHK cell line. Murine norovirus (MNV) used for seeding was MNV-1 propagated in house using the RAW 264.7 cell line. Three spike levels of HAV were used for this platform extension study: Low (0.1 PFU/g of HAV), Medium (1.0 PFU/g HAV) and uninoculated. MNV was inoculated in all samples at 10^2 PFU/g.

Negative Finfish Matrix

The protocol "Concentration and Extraction of Enteric Viruses from Scallops and Tuna" (BAM Chapter 26, Section A4) was performed to obtain negative finfish extracts. The resulting template was used to test the stability of the assay with negative food matrix.

Results

Table F18 (A-C). Intra- Assay Variability of the HAV Detection Assay. Three 10-fold dilutions of 9 replicates of HAV RNA were tested (10, 1, and 0.1 PFU/reaction) in 3 µl reactions. This experiment was repeated in three independent trials.

A.

	Trial 1					
	Smart Cycler			**AB 7500**		
Spike Level	**High**	**Medium**	**Low**	**High**	**Medium**	**Low**
Mean C_t*	28.28	31.19	34.61	28.9	33.06	36.21
SD	0.16	0.44	0.37	0.18	0.12	0.25
SE	0.05	0.14	0.12	0.05	0.38	0.08
Amplification efficiency	107%			93%		
r^2	0.99			0.99		

B.

	Trial 2					
	Smart Cycler			**AB 7500**		
Spike Level	**High**	**Medium**	**Low**	**High**	**Medium**	**Low**
Mean C_t*	28.24	31.04	34.88	25.83	29.49	33.03
SD	0.23	0.39	0.42	0.49	0.45	0.39
SE	0.07	0.12	0.13	0.15	0.143	0.12
Amplification efficiency	100%			90%		
r^2	0.99			0.99		

C.

	Trial 3					
	Smart Cycler			**AB 7500**		
Spike Level	**High**	**Medium**	**Low**	**High**	**Medium**	**Low**
Mean C_t*	28.38	32.06	35.27	29.47	32.64	36.05
SD	0.84	0.37	0.22	0.15	0.30	0.31
SE	0.26	0.12	0.07	0.049	0.09	0.10
Amplification efficiency	95%			101%		
r^2	0.99			0.99		

*Cycle threshold averaged for 9 replicates of 3 µl reactions

E2 Mean Efficiency: Smart Cycler 101%; $r^2 = 0.99$
AB7500 95%; $r^2 = 0.99$

I**ntra-Assay Reproducibility (Table F18A-18C):** Nine reactions of varying low (C_t 33-36) medium (C_t 29-32) and high (C_t 25-29) concentrations were tested in triplicate on the same run for the Smart Cycler and AB7500. The results show excellent reproducibility and amplification efficiencies that fall within the acceptable range (90-110 %).

Table F19. Limit of Detection (LOD)/Limit of Quantitation (LOQ)- HAV175/18*f*

PFU/rxn[α]	Ave. C_t	SD*	Pos/Total
0.11 PFU	36.21	0.23	9/9
0.01 PFU	40.72	0.97	8/9
0.001 PFU	42.97	0.42	2/9

***** Standard deviation of positive samples only.
α PFU; fraction of viral particles able to infect susceptible in cell culture under idealized in vitro conditions
LOQ= 0.11 PFU/rxn
LOD= 0.001 PFU/rxn

Table F20. No Template Control (PCR Grade Water)

Sample	HAV C_t	HAV IAC C_t	Sample	HAV C_t	HAV IAC C_t	Sample	HAV C_t	HAV IAC C_t	Sample	HAV C_t	HAV IAC C_t
NTC	0.00	24.23	NTC	0.00	24.20	NTC	0.00	24.20	NTC	0.00	24.18
NTC	0.00	24.18	NTC	0.00	24.11	NTC	0.00	24.15	NTC	0.00	24.15
NTC	0.00	24.12	NTC	0.00	24.13	NTC	0.00	24.08	NTC	0.00	24.10
NTC	0.00	24.05	NTC	0.00	24.05	NTC	0.00	24.03	NTC	0.00	24.07
NTC	0.00	24.09	NTC	0.00	24.01	NTC	0.00	23.95	NTC	0.00	24.18
NTC	0.00	24.05	NTC	0.00	23.95	NTC	0.00	24.00	NTC	0.00	24.06
NTC	0.00	24.02	NTC	0.00	24.00	NTC	0.00	24.00	NTC	0.00	24.02
NTC	0.00	24.05	NTC	0.00	23.97	NTC	0.00	23.99	NTC	0.00	24.02
NTC	0.00	24.09	NTC	0.00	24.04	NTC	0.00	24.03	NTC	0.00	24.11
NTC	0.00	24.16	NTC	0.00	24.12	NTC	0.00	24.11	NTC	0.00	24.20
NTC	0.00	24.24	NTC	0.00	24.17	NTC	0.00	24.19	NTC	0.00	24.19
NTC	0.00	24.36	NTC	0.00	24.37	NTC	0.00	24.20	NTC	0.00	24.19
NTC	0.00	24.16	NTC	0.00	24.24	NTC	0.00	24.20	NTC	0.00	24.27
NTC	0.00	24.16	NTC	0.00	24.11	NTC	0.00	24.13	NTC	0.00	24.16
NTC	0.00	24.10	NTC	0.00	24.05	NTC	0.00	24.01	NTC	0.00	24.16
NTC	0.00	24.07	NTC	0.00	24.00	NTC	0.00	24.02	NTC	0.00	24.05
NTC	0.00	24.00	NTC	0.00	23.97	NTC	0.00	24.00	NTC	0.00	24.01
NTC	0.00	24.00	NTC	0.00	23.92	NTC	0.00	24.00	NTC	0.00	24.07
NTC	0.00	23.96	NTC	0.00	23.95	NTC	0.00	23.98	NTC	0.00	24.01
NTC	0.00	24.00	NTC	0.00	23.97	NTC	0.00	23.98	NTC	0.00	24.01
NTC	0.00	24.04	NTC	0.00	23.99	NTC	0.00	24.06	NTC	0.00	24.12
NTC	0.00	24.05	NTC	0.00	24.05	NTC	0.00	24.11	NTC	0.00	24.16
NTC	0.00	24.15	NTC	0.00	24.09	NTC	0.00	24.15	NTC	0.00	24.18
NTC	0.00	24.26	NTC	0.00	24.20	NTC	0.00	24.28	POS	29.05	24.70

Table F21. Negative Finfish Matrix

Sample	HAV C_t	HAV IAC C_t	Sample	HAV C_t	HAV IAC C_t	Sample	HAV C_t	HAV IAC C_t	Sample	HAV C_t	HAV IAC C_t
Tuna 1	0.00	24.58	Tuna 2	0.00	24.42	Tuna 3	0.00	24.41	Tuna 4	0.00	24.50
Tuna 1	0.00	24.63	Tuna 2	0.00	24.38	Tuna 3	0.00	24.37	Tuna 4	0.00	24.50
Tuna 1	0.00	24.54	Tuna 2	0.00	24.44	Tuna 3	0.00	24.36	Tuna 4	0.00	24.46
Tuna 1	0.00	24.64	Tuna 2	0.00	24.40	Tuna 3	0.00	24.41	Tuna 4	0.00	24.46
Tuna 1	0.00	24.52	Tuna 2	0.00	24.47	Tuna 3	0.00	24.36	Tuna 4	0.00	24.48
Tuna 1	0.00	24.55	Tuna 2	0.00	24.44	Tuna 3	0.00	24.39	Tuna 4	0.00	24.52
Tuna 1	0.00	24.43	Tuna 2	0.00	24.46	Tuna 3	0.00	24.43	Tuna 4	0.00	24.55
Tuna 1	0.00	24.54	Tuna 2	0.00	24.44	Tuna 3	0.00	24.40	Tuna 4	0.00	24.54
Tuna 1	0.00	24.60	Tuna 2	0.00	24.38	Tuna 3	0.00	24.35	Tuna 4	0.00	24.64
Tuna 1	0.00	24.52	Tuna 2	0.00	24.34	Tuna 3	0.00	24.42	Tuna 4	0.00	24.57
Tuna 1	0.00	24.53	Tuna 2	0.00	24.38	Tuna 3	0.00	24.40	Tuna 4	0.00	24.51
Tuna 1	0.00	24.50	Tuna 2	0.00	24.31	Tuna 3	0.00	24.34	Tuna 4	0.00	24.57
Tuna 1	0.00	24.50	Tuna 2	0.00	24.38	Tuna 3	0.00	24.33	Tuna 4	0.00	24.62
Tuna 1	0.00	24.58	Tuna 2	0.00	24.41	Tuna 3	0.00	24.44	Tuna 4	0.00	24.49
Tuna 1	0.00	24.55	Tuna 2	0.00	24.42	Tuna 3	0.00	24.39	Tuna 4	0.00	24.54
Tuna 1	0.00	24.55	Tuna 2	0.00	24.46	Tuna 3	0.00	24.36	Tuna 4	0.00	24.51
Tuna 1	0.00	24.52	Tuna 2	0.00	24.29	Tuna 3	0.00	24.43	Tuna 4	0.00	24.58
Tuna 1	0.00	24.48	Tuna 2	0.00	24.29	Tuna 3	0.00	24.38	Tuna 4	0.00	24.65
Tuna 1	0.00	24.38	Tuna 2	0.00	24.24	Tuna 3	0.00	24.43	Tuna 4	0.00	24.77
Tuna 1	0.00	24.43	Tuna 2	0.00	24.34	Tuna 3	0.00	24.32	Tuna 4	0.00	24.52
Tuna 1	0.00	24.43	Tuna 2	0.00	24.37	Tuna 3	0.00	24.46	Tuna 4	0.00	24.55
Tuna 1	0.00	24.49	Tuna 2	0.00	24.30	Tuna 3	0.00	24.42	Tuna 4	0.00	24.61
Tuna 1	0.00	24.49	Tuna 2	0.00	24.48	Tuna 3	0.00	24.56	NTC	0.00	24.48
Tuna 1	0.00	24.54	Tuna 2	0.00	24.31	Tuna 3	0.00	24.52	POS	29.59	24.57

Table F22. Frozen Strawberry Matrix on Smart Cycler and AB 7500

Strawberry Matrix	Medium 5 PFU/g	Low 0.5 PFU/g	Uninoculated 0 PFU/g
Smart Cycler	3 of 3	1 of 3	0 of 2
Smart Cycler – Percent Positive	100	33	0
AB 7500	3 of 3	3 of 3	0 of 2
AB 7500 – Percent Positive	100	100	0

Conclusion

The results of the validations for the HAV RT-qPCR detection assay demonstrate the assay is sensitive, specific, reproducible and robust. Our conclusion is that this assay can be used for the detection of HAV in RNA preparations obtained from any food matrix. This assay is ready to be incorporated into the Bacteriological Analytical Manual and ongoing Office of Regulatory Affairs Field Assignments.

Data Analysis for HAV Detection Assay and Control Exclusion Assay

The HAV multiplex RT-qPCR assay is used to detect HAV RNA in food matrices. The Cy5 and Texas Red channels correlating to the HAV and the IAC targets, respectively. Positive HAV detection occurs when the primary fluorescence curve crosses the threshold for HAV and the IAC is positive. For HAV samples where HAV is detected, the HAV laboratory strain has sequence identities that can be used to differentiate between the laboratory and majority of wild-type strains. The Control Exclusion Assay uses a RT-qPCR assay for the differentiation of the HAV laboratory control strain. All primers and probes should be hydrated to a concentration of 100 µM using primer TE prior to making primer and probe mixes.

Data Interpretation for HAV RT-qPCR 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 the detection HAV.
3. Samples are invalid if:
    a. The negative RT-qPCR control sample demonstrates positive results crossing the Cy5 or if the IAC is negative, repeat the RT-qPCR assay.
    b. The average of the IAC C_t values for the sample replicates are more than 4.0 C_ts greater than the Negative Control IAC C_t value, repeat the RT-qPCR assay using remaining RNA or RNA from a newly extracted saved tube with a 1 µl RT-qPCR reaction in triplicate. If the 1 µl template reactions yield an average IAC Ct value greater than 4.0 Ct higher than the Negative Control IAC Ct value, repeat the sample analysis from the beginning using additional food sample. With the new sample, the concentrates will be split into 5 tubes and complete RT-qPCR with 1 µl reactions in triplicate.

Note: A positive sample is a result that demonstrates log amplification. Log amplification can be viewed as a graph on the AB 7500 platforms. If the sample does not exhibit log amplification and crosses the threshold, the RT-qPCR reaction should be repeated. Refer to figures in Appendix E for appropriate amplification curves.

Data Analysis for Control Exclusion Assay (CEA)

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

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

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

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

Assessment of CEA positive samples

The HAV CEA probe targets the HAV laboratory control strain and typically will not generate a positive RT-qPCR result unless wild-type HAV is present in titers $\geq 10^3$, including positive controls from culture (genomic) RNA. All positive samples will need to be analyzed by 3% agarose gel or on a genetic bioanalyzer such as an Agilent Tapestation or equivalent genetic bioanalyzer. If a genetic bioanalyzer will be used, manufacturer's instructions for analysis of product should be followed. Detailed instructions for the 3% agarose gel analysis are listed below. The HAV laboratory control strain will yield a product size of 180 bp while other strains (wild-type) will yield a product size of 169 bp (Figure F1). You will also observe the internal control band size of 148 bp. On rare occasions, amplification of wild-type strains may produce bands with sizes ranging between 180 bp and 160 bp but not 169 bp; these bands should be excised and sequenced to confirm HAV RNA.

3.0% Agarose Gel Analysis:

1. Prepare 1X TAE buffer.
2. Rinse and dry the gel tray.
3. Place gel tray in gel caster; adjust to fix tray and level.
4. Add comb to the first slot of the tray, making sure the comb fits securely in the slots.
5. Pour 200 ml of 1X TAE buffer into 250 ml glass bottle with magnetic stirrer.
6. Weigh 6 g of agarose and add to 200 ml buffer.
7. Shake briefly and microwave for 1-3 min (until the agarose is completely dissolved and there is a nice momentary boil).
8. Place on stirrer and let cool for 5-10 min. Add 4 µl of ethidium bromide, stir for additional 30 sec.
9. Carefully pour the 200 mL agarose into the casting tray.

Note: The depth of the gel should be 5 mm so the volume of the gel may need to be adjusted depending on the size of the gel casting tray.

10. Allow the gel to solidify (about 1 hr); then remove the comb.

11. Add 1X TAE buffer to electrophoresis chamber; careful not to overfill.

12. Insert the casting tray (with the gel on it) into the electrophoresis chamber closest to the negative (black) electrode. DNA is negatively charged. During electrophoresis, it will migrate from the negative (black) to the positive (red) electrode.

13. If gel is not covered with enough 1X TAE, gradually add 1X TAE to electrophoresis chamber until the buffer just covers the top of the gel.

14. Add 0.5 µl of loading dye to each sample. Skip first and last well and load 15 µl of each sample to individual wells, then add the 100 bp ladder to the first and last well, taking care not to puncture the well bottoms. Do not attempt to load a sample if there is an air bubble in your pipet tip. Also, if a well has an air bubble in it, push it out using a clean tip before loading a sample in it.

15. Attach lid; make sure cords are correctly plugged into the power supply (red to red, black to black).

16. Plug in the power supply.

17. Turn the power on and adjust to 100 volts.

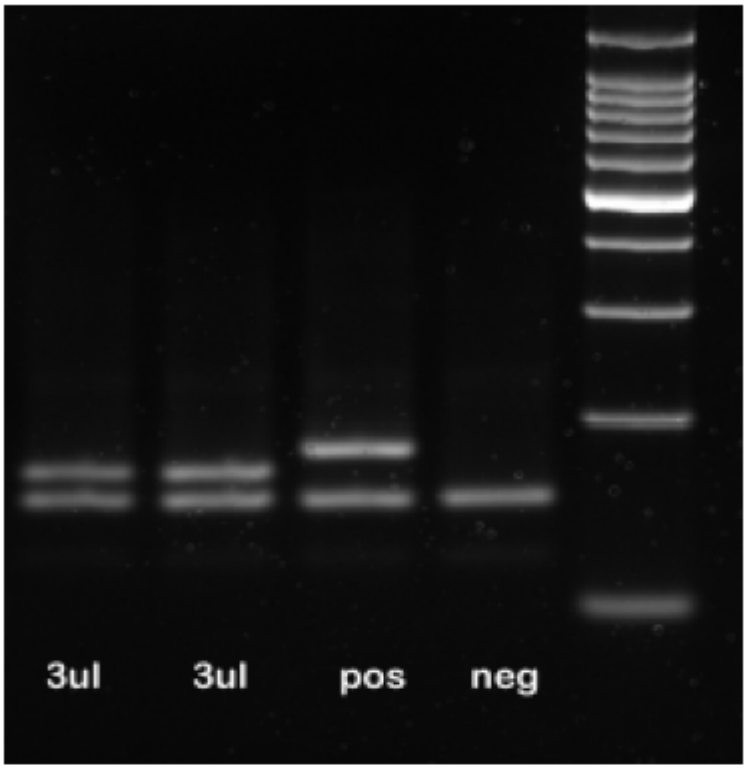
18. Electrophorese (run) the gel for 2 hrs.

19. Shut off the power supply, unplug the leads, and unplug the power supply.

20. Lift the gel casting tray from the chamber.

21. Place gel in gel imager and analyze according to manufacturer's instructions.

Figure F1. 3% Low-melt temp agarose gel with positive wild-type and controls



Lane 1—3ul sample with 148 bp IAC band and 169 bp wild-type band

Lane 2—3ul sample with 148 bp IAC band and 169 bp wild-type band

Lane 3—IAC 148 bp band and positive control 180 bp band

Lane 4—negative

Lane 5—100bp ladder

Supplemental Material for HAV Detection Assay

Table F23. HAV, MNV, and Norovirus Buffer Mix Setup*

Reagents	**Volume (50 mM MgCl)**	**Volume (25 mM MgCl)**
DNase/RNase free H_2O	1760 µl	1610 µl
5X Buffer	1000 µl	1000 µl
MgCl	150 µl	300 µl
dNTPs	200 µl	200 µl

*Made with components from Qiagen One-Step RTqPCR kit and PCR grade water

Table F24. Enzyme Mix Setup

Component	**Volume**
One-Step Qiagen enzyme	~200 µL
Superase-IN (10000U)	50 µL

Table F25. HAV Primer Mix Setup*

Primers/H_2O	**Volume**
GAR2F	30 µl
GAR1R	30 µl
IC46F	7.44 µl
IC194R	7.44 µl
DNase/RNase water	725.12 µl

*Made with 100 µM primers stocks and PCR grade water

Table F26. HAV Probe Mix

Probe/H_2O	**Volume**
HAVP	30 µl
ICP	22.5 µl
DNase/RNase water	547.10 µl

*Made with 100 µM probe stocks and PCR grade water

Supplemental Material for Control Exclusion Assay

Table F27. CEA Buffer Mix Setup*

Reagents	**Volume (50 mM MgCl)**	**Volume (25 mM MgCl)**
DNase/RNase free H_2O	1660 µl	1360 µl
5X Buffer	1000 µl	1000µl
$MgCl_2$	300 µl	600 µl
dNTPs	150 µl	150 µl

*Made with components from Qiagen One-Step RT-qPCR kit and PCR grade water

Table F28. CEA Primer Mix Setup*

Primers/H_2O	**Volume**
HAVCROF	7.44 µl
JWCROR	7.44 µl
IC46F	7.44 µl
IC194R	7.44 µl
DNase/RNase water	770.24 µl

*Made with 100 µM primers stocks and PCR grade water

Table F29. CEA Probe Mix

Probe/H_2O	**Volume**
JWCROP	10 µl
ICP	15 µl
DNase/RNase water	375 µl

*Made with 100 µM probe stocks and PCR grade water

Appendix G: Validation Data, Data Analysis, and Supplemental Material for the Norovirus Genogroup I and Genogroup II Detection Assay

The RT-qPCR detection assay includes oligonucleotide primers and dual-labeled hydrolysis (TaqMan®-style) probes for the *in vitro* detection of norovirus GI and GII. The norovirus primers target the ORF 1 and 2 junction of the norovirus genome. This region of the genome is highly variable, and these primers and probes have previously been shown to detect multiple strains of norovirus GI and GII (Kageyama et al., 2003). This assay also incorporates an RNA internal amplification control (IAC) to monitor any potential matrix-derived inhibitory effects. All primers and probes should be hydrated to a concentration of 100 µM using primer TE prior to making primer and probe mixes.

Single Laboratory Validation of Norovirus RT-qPCR Detection Assay

The norovirus detection assay has been effectively optimized and has been used extensively in research and outbreak analysis for norovirus (DePaola et al., 2010; Woods et al., 2016). The single laboratory validation (SLV) followed the FDA Guidelines for the Validation of Analytical Methods for the Detection of Microbial Pathogens in Foods, 2nd Ed (2015). The SLV was conducted at GCSL in accordance with a level 2 validation on the Smart Cycler and AB 7500 platforms. Results are presented separately for each instrument platform. For the RT-qPCR assay for the SLV inclusivity, 17 different strains of GI or GII were used (Table G1). For the exclusivity assay, 19 different strains of enteric bacteria or viruses were used (Table G2). In addition, known positive clinical samples from state and international laboratories were tested with the norovirus multiplex assay yielding 100% detection on the Smart Cycler and AB 7500 platforms (Table G3 and G10). The amplification efficiencies ranged from 91% to 99% on the Smart Cycler and 95% to 99% for the AB 7500 (Tables G4 and G11). There was no inhibition in the assay with the addition of the internal amplification control (IAC) or competitive RNA with and without norovirus GI or GII (Table G5 and G12). On the Smart Cycler, the dynamic range of the assay was 7 logs (Figure G1) and the LOQ and LOD were 1 and 10 genomic copies/reaction, respectively (Table G7). For the AB 7500 platform, the dynamic range of the assay was 6 and 7 logs for GI and GII respectively (Figure G4) and the LOQ and LOD were 1 and 10 genomic copies/reaction, respectively (Table G13).

Sample Preparation

Viral Nucleic Acid Templates (for inclusivity/exclusivity and competitive RNA testing)

RNA was isolated from stock suspensions and diluted for inclusive and exclusive viruses (Tables G1 and G2) and stool samples using the QIAamp Viral RNA Mini Kit (Qiagen) protocol for cell culture. The RNA was eluted from the spin columns with 60 µl AVE elution buffer (provided in kit) and stored at -80 ºC until used.

Bacterial Templates (for exclusivity testing)

DNA templates were prepared by transferring 1 ml of overnight Tryptic Soy Broth culture to a microcentrifuge tube and centrifuging at 12,000 x *g* for 3 min. The supernatant was removed and the pellet resuspend in 1 ml 0.85% NaCl. The tube was centrifuged at 12,000 x *g* for 3 min. The supernatant was removed and the pellet was completely resuspend in 1 ml sterile water. The tube was placed in a water bath or heat block maintained at 100 °C for 10 min. Following heating, the tube was centrifuge

12,000 x *g* for 1 min, and the supernatant was removed to a new microcentrifuge tube. This bacterial extract was frozen at -20 °C until used.

SLV Results for the Norovirus Detection Assay on the Smart Cycler

Table G1. Inclusivity of the norovirus detection assay

Genotype/Strain	Source	Norovirus Average C_t*	SD	IAC Average C_t	SD	Frequency of Result
GII.4 Henry	clinical	34.87	0.34	30.99	0.20	6 of 6
GII.4 Grimsby	clinical	31.55	0.70	23.72	0.23	6 of 6
GII.4 Minerva	clinical	27.12	0.66	25.21	0.62	6 of 6
GII.4 Hunter	clinical	26.36	0.57	24.51	0.14	6 of 6
GII.4 Osaka	clinical	25.38	0.37	23.82	0.37	6 of 6
GII.4 New Orleans	clinical	24.14	0.66	23.72	0.84	6 of 6
GII.1	clinical	20.63	0.38	24.22	0.35	6 of 6
GII.6A	clinical	25.78	0.33	25.15	0.32	6 of 6
GII.12	clinical	21.69	0.63	25.69	0.42	6 of 6
GII.16	clinical	35.62	0.34	24.41	0.47	6 of 6
GI.6A	clinical	33.97	0.39	26.18	0.29	6 of 6
GI.1	clinical	33.41	0.55	27.17	0.15	6 of 6
GI.2	clinical	29.09	0.32	26.34	0.23	6 of 6
GI.2 Constellation	clinical	27.36	0.18	26.48	0.14	6 of 6
GI.4	clinical	27.67	1.07	26.55	0.17	6 of 6
GI.3C	clinical	26.72	1.08	26.18	0.13	6 of 6
GI.3B	clinical	37.39	0.56	26.10	0.34	6 of 6

*Average C_t value of 6 replicates.

Table G2. Exclusivity testing of the norovirus detection assay

Strain	Source	Results	Frequency
Murine norovirus	David Kingsley – cell culture	negative	6 of 6
San Miguel Sea Lion Virus serogroup 17	Dr. Alvin Smith, University of Oregon	negative	6 of 6
Astrovirus	clinical	negative	6 of 6
HAS-15	ATCC VR2281	negative	6 of 6
Adenovirus 41	clinical	negative	6 of 6
Adenovirus 40	clinical	negative	6 of 6
Sabine poliovirus	cell culture	negative	6 of 6
Shigella sonnei	ATCC 9290	negative	6 of 6
Paraechovirus	clinical	negative	6 of 6
Echovirus 30	clinical	negative	6 of 6
Enterovirus 90	clinical	negative	6 of 6
Vibrio parahaemolyticus	Oyster extract	negative	6 of 6
Vibrio spp.	ATCC 14035	negative	6 of 6
Salmonella enterica	ATCC 9700	negative	6 of 6
Rotavirus	ATCC VR2018	negative	6 of 6
Listeria monocytogenes	ATCC 7646	negative	6 of 6
HAV HM175f	cell culture	negative	6 of 6
Coxsackievirus A1	clinical	negative	6 of 6
Norovirus GIV	IDT RNA transcript	negative	6 of 6

Table G3. Specificity testing of norovirus detection assay with clinical samples

	GI	IAC	GII
1	42.40	29.10	25.57
2	0.00	28.70	32.89
3	42.19	30.43	34.94
4	0.00	33.62	40.76
5	47.22	30.08	25.48
6	0.00	32.05	31.86
7	46.05	27.76	28.53
8	0.00	29.30	28.01
9	0.00	29.09	26.45
10	0.00	28.94	42.54
11	42.71	28.27	0.00
12	26.57	28.46	0.00
13	0.00	27.86	0.00
14	33.34	27.27	0.00
15	34.87	28.27	0.00
16	39.88	27.39	0.00
17	37.63	27.05	0.00
18	28.19	27.96	0.00
19	34.41	27.31	0.00
20	37.92	28.42	0.00
21	0.00	25.13	0.00
22	0.00	28.05	0.00
23	0.00	30.84	0.00
24	0.00	27.74	0.00
Positive	29.30	27.38	28.73
Negative	0.00	27.38	0.00

Table G4. Norovirus RT-qPCR Intra-assay Variability: three 100-fold dilutions of GI and GII RNA, 9 replicates per dilution

	Trial 1						**Trial 2**					
C_t*	**High**		**Med**		**Low**		**High**		**Med**		**Low**	
Template	GI	GII	GI	GII	GI	GII	GI	GII	GI	GII	GI	GII
Mean	17.69	22.47	24.19	29.04	31.79	37.30	17.77	22.27	24.10	28.96	31.87	36.62
SD	0.26	0.41	0.52	0.48	0.29	1.19	1.02	1.18	0.40	0.32	1.28	1.16
SE	0.08	0.13	0.17	0.16	0.09	0.45	0.34	0.39	0.13	0.10	0.42	0.44
Mean Amplification Efficiency	95%						99%					
r^2	0.997						0.998					
	Trial 3						**Trial 4**					
C_t*	**High**		**Med**		**Low**		**High**		**Med**		**Low**	
Template	GI	GII	GI	GII	GI	GII	GI	GII	GI	GII	GI	GII
Mean	17.46	22.31	23.72	28.55	31.35	35.80	17.74	21.99	24.25	28.54	32.07	36.01
SD	0.37	0.60	0.47	0.40	0.62	0.39	0.104	0.16	0.171	0.26	0.377	0.63
SE	0.12	0.29	0.16	0.13	0.21	0.13	0.035	0.05	0.057	0.09	0.142	0.21
Mean Amplification Efficiency	91%						98%					
r^2	0.997						0.997					
	Trial 5											
C_t*	**High**		**Med**		**Low**							
Template	GI	GII	GI	GII	GI	GII						
Mean	17.82	21.79	23.97	28.24	31.81	35.62						
SD	0.32	0.17	0.29	0.25	0.32	1.01						
SE	0.11	0.06	0.10	0.08	0.11	0.33						
Mean Amplification Efficiency	95%											
r^2	0.996											

*From 9 replicates
Mean Efficiency: 96%; $r^2 = 0.997$

I**ntra-assay reproducibility:** Five trials with the multiplex assay of low (C_t 31-37) medium (C_t 23-28) and high (C_t 17-21) concentrations were tested in 9 replicates. The results show excellent reproducibility.

Inter-assay reproducibility: The C_t value of the daily positive control was analyzed over a period of 5 days on 6 different, calibrated, Smart Cycler blocks. The results show consistent reproducibility over the period of time on the same sample with mean amplification efficiencies and r^2 values of 96% and 0.997, respectively.

Table G5. Detection of norovirus GI in the presence of IAC and GII

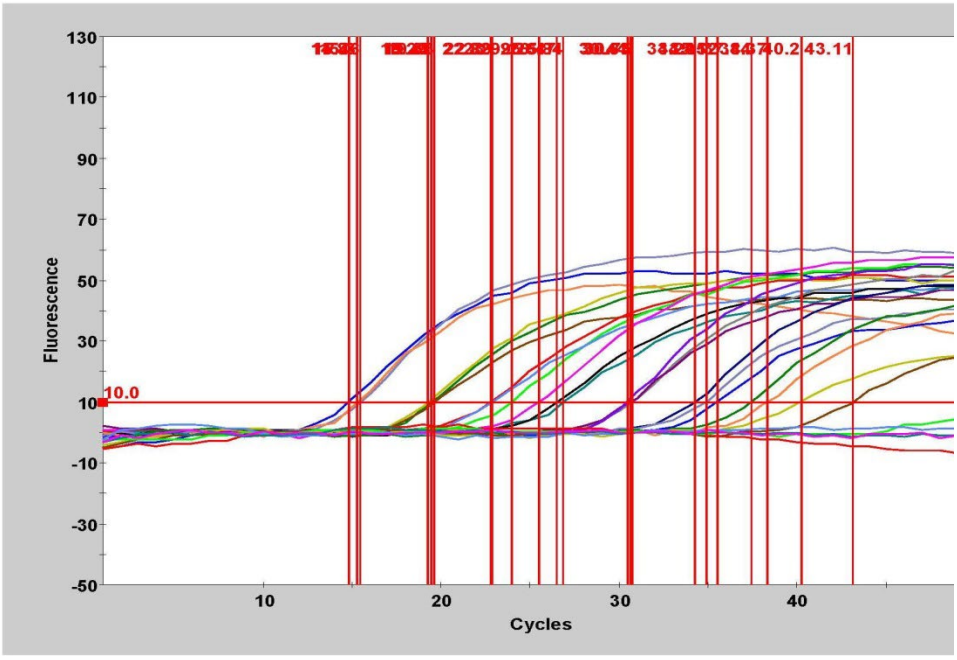
		Without IAC		With IAC			
		GI Average C_t	**GI SD**	**IAC Average C_t**	**IAC SD**	**GI Average C_t**	**GI SD**
With GII	**GI -2 Dilution**	18.29	0.19	30.32	0.60	18.06	0.54
	GI -3 Dilution	21.77	0.35	28.69	0.21	21.18	0.15
	GI -4 Dilution	24.83	0.49	28.14	0.20	24.29	0.26
Without GII	**GI -2 Dilution**	18.64	0.92	34.39	0.55	18.13	0.30
	GI -3 Dilution	21.07	0.16	31.46	0.42	20.80	0.29
	GI -4 Dilution	24.08	0.26	29.03	0.32	23.54	0.17

Table G6. Detection of norovirus GII in the presence of IAC and GI

		Without IAC		With IAC			
		GII Average C_t	**GII SD**	**IAC Average C_t**	**IAC SD**	**GII Average C_t**	**GII SD**
With GI	**GII -2 Dilution**	22.03	0.28	28.98	0.20	21.68	0.52
	GII -3 Dilution	25.60	0.20	28.44	0.34	25.35	0.46
	GII -4 Dilution	29.21	0.24	28.62	0.21	29.29	0.26
Without GI	**GII -2 Dilution**	22.00	0.52	28.77	0.42	22.29	0.54
	GII -3 Dilution	25.57	0.24	28.38	0.34	25.35	0.48
	GII -4 Dilution	28.93	0.16	28.14	0.18	28.78	0.29

Figure G1 (A-B). Dynamic range of the norovirus GI and GII detection assay

GIA. GI



..

GIB. GII

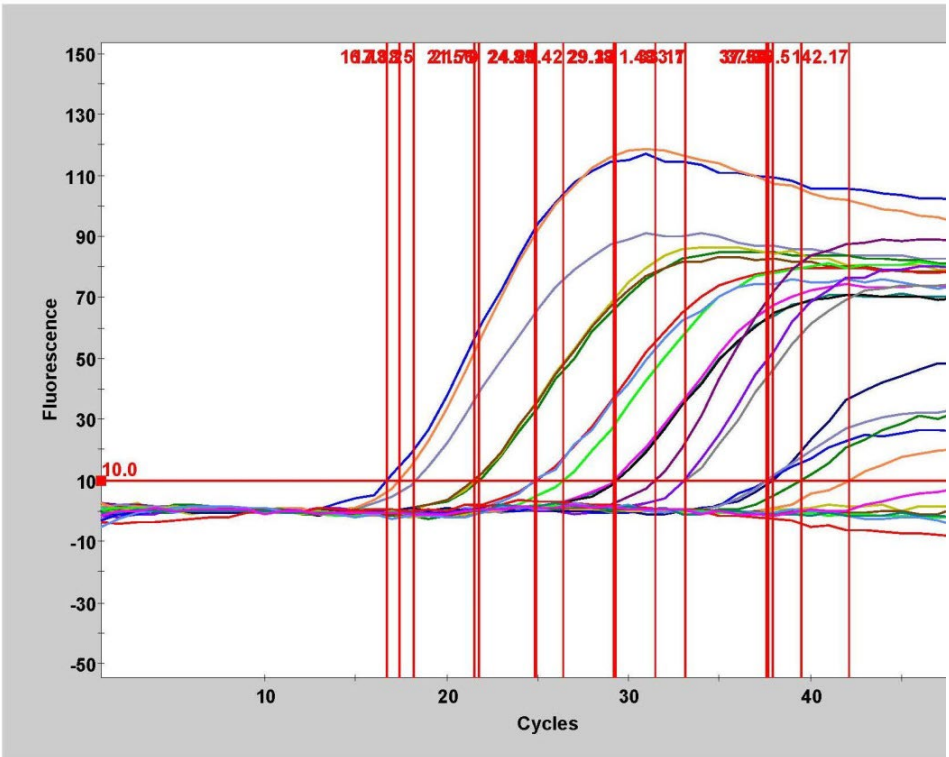
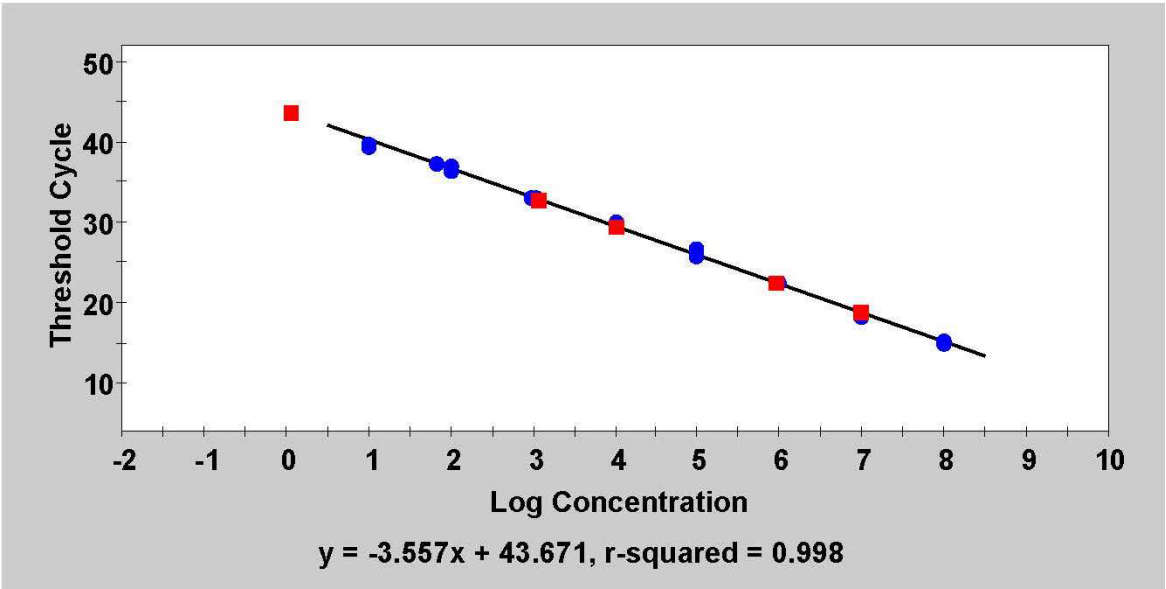


Figure G2 (A-B). Standard curves 2

G2A. GI reaction efficiency of 91%



G2B. GII efficiency of 90%

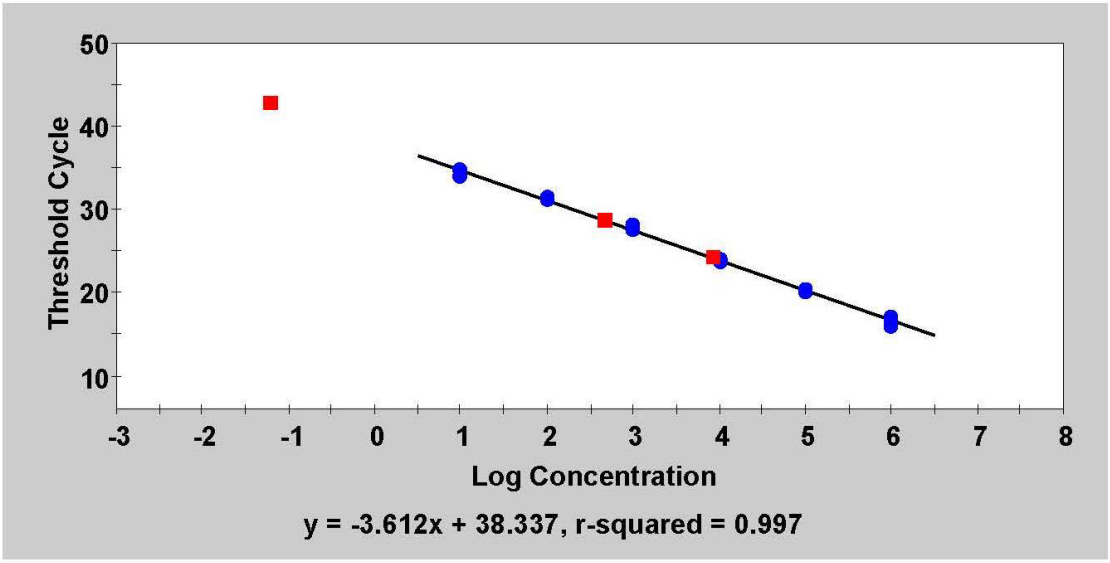


Table G7. LOD / LOQ detection assay using GI.1 and GII.4

	Norovirus GI			Norovirus GII		
Genomic copies/rxn	**Average C_t**	**SD***	**Pos/Total**	**Average C_t**	**SD***	**Pos/Total**
100	30.64	0.12	6 of 6	31.08	1.08	6 of 6
10	35.58	1.68	6 of 6	34.80	0.81	6 of 6
1	41.66	2.05	2 of 6	42.78	3.60	3 of 6

*standard deviation of positive samples only

LOQ = 10 genomic copies
LOD = 1 genomic copies

SLV Results for the Norovirus Detection Assay on the AB 7500

Table G8. Inclusivity of the norovirus detection assay

Genotype/Strain	Source	Norovirus Average C_t*	SD	IAC Average C_t	SD	Frequency
GII.4 Henry	clinical	33.88	0.34	28.66	0.20	6 of 6
GII.4 Grimsby	clinical	30.26	0.70	23.72	0.23	6 of 6
GII.4 Minerva	clinical	26.34	0.66	25.21	0.62	6 of 6
GII.4 Hunter	clinical	25.21	0.57	24.51	0.14	6 of 6
GII.4 Osaka	clinical	24.59	0.37	23.82	0.37	6 of 6
GII.4 New Orleans	clinical	23.31	0.14	23.72	0.35	6 of 6
GII.1	clinical	36.35	0.38	24.22	0.32	6 of 6
GII.6A	clinical	25.08	0.33	25.15	0.42	6 of 6
GII.12	clinical	20.18	0.63	25.69	0.47	6 of 6
GII.16	clinical	34.04	0.34	24.41	0.29	6 of 6
GI.6A	clinical	33.97	0.39	26.18	0.15	6 of 6
GI.1	clinical	33.41	0.55	27.17	0.23	6 of 6
GI.2	clinical	29.09	0.32	26.34	0.14	6 of 6
GI.2 Constellation	clinical	27.36	0.18	26.48	0.17	6 of 6
GI.4	clinical	27.67	1.07	26.55	0.13	6 of 6
GI.3C	clinical	26.72	1.08	26.18	0.34	6 of 6
GI.3B	clinical	37.39	0.56	26.10	0.31	6 of 6

*Average of 6 replicates

Table G9. Exclusivity testing of the norovirus detection assay

Strain	Source	Results	Frequency
Murine norovirus	David Kingsley – cell culture	negative	6 of 6
San Miguel Sea Lion Virus serogroup 17	Dr. Alvin Smith, University of Oregon	negative	6 of 6
Astrovirus	clinical	negative	6 of 6
HAS-15	ATCC VR2281	negative	6 of 6
Adenovirus 41	clinical	negative	6 of 6
Adenovirus 40	clinical	negative	6 of 6
Sabine poliovirus	cell culture	negative	6 of 6
Shigella sonnei	ATCC 9290	negative	6 of 6
Paraechovirus	clinical	negative	6 of 6
Echovirus 30	clinical	negative	6 of 6
Enterovirus 90	clinical	negative	6 of 6
Vibrio parahaemolyticus	Oyster extract	negative	6 of 6
Vibrio spp.	ATCC 14035	negative	6 of 6
Salmonella enterica	ATCC 9700	negative	6 of 6
Rotavirus	ATCC VR2018	negative	6 of 6
Listeria monocytogenes	ATCC 7646	negative	6 of 6
HAV HM175f	cell culture	negative	6 of 6
Coxsackievirus A1	clinical	negative	6 of 6
Norovirus GIV	IDT RNA transcript	negative	6 of 6

Table G10. Specificity testing of norovirus detection assay with clinical samples

	GI	IAC	GII
1	0.00	26.63	23.67
2	0.00	26.81	31.32
3	0.00	26.37	33.27
4	0.00	28.15	34.71
5	0.00	27.15	23.27
6	42.87	27.45	28.93
7	0.00	26.25	26.83
8	43.79	27.23	26.26
9	0.00	26.94	24.73
10	0.00	26.73	40.08
11	41.90	26.51	0.00
12	26.16	26.97	0.00
13	0.00	26.26	0.00
14	32.26	26.28	0.00
15	32.90	26.00	0.00
16	38.52	26.90	40.61
17	35.59	26.43	0.00
18	27.72	26.41	0.00
19	34.03	26.52	0.00
20	37.86	26.38	0.00
21	0.00	26.84	0.00
22	0.00	26.73	0.00
23	0.00	26.65	0.00
24	0.00	26.30	0.00
Positive	28.12	26.29	27.19
Negative	0.00	26.41	0.00

Table G11. Norovirus RT-qPCR Intra Assay Variability: three 100-fold dilutions of GI and GII RNA

	Trial 1						Trial 2					
C_t*	**High**		**Med**		**Low**		**High**		**Med**		**Low**	
Template	GI	GII	GI	GII	GI	GII	GI	GII	GI	GII	GI	GII
Mean	18.05	21.90	24.62	28.59	32.20	36.24	17.63	21.37	24.30	28.18	31.91	35.47
SD	0.17	0.17	0.10	0.10	0.14	0.38	0.07	0.14	0.09	0.24	0.12	0.81
SE	0.05	0.06	0.03	0.03	0.02	0.13	0.02	0.04	0.12	0.08	0.04	0.27
Mean Amplification Efficiency	97%						95%					
r^2	0.996						0.995					

	Trial 3						Trial 4					
C_t*	**High**		**Med**		**Low**		**High**		**Med**		**Low**	
Template	GI	GII	GI	GII	GI	GII	GI	GII	GI	GII	GI	GII
Mean	17.48	21.24	24.24	28.09	31.84	35.44	17.46	21.39	24.04	27.96	31.48	35.21
SD	0.15	0.09	0.13	0.26	0.27	0.40	0.09	0.12	0.10	0.13	0.28	0.44
SE	0.05	0.31	0.27	0.40	0.09	0.13	0.03	0.04	0.03	0.03	0.09	0.14
Mean Amplification Efficiency	98%						98%					
r^2	0.998						0.997					

	Trial 5					
C_t*	**High**		**Med**		**Low**	
Template	GI	GII	GI	GII	GI	GII
Mean	17.37	21.31	23.96	27.95	31.73	35.01
SD	0.17	0.17	0.19	0.23	0.45	0.31
SE	0.05	0.06	0.06	0.08	0.14	0.10
Mean Amplification Efficiency	99%					
r^2	0.996					

*From 9 replicates
Mean Efficiency: 97%; $r^2 = 0.997$

I**ntra-Assay Reproducibility:** Five trials with the multiplex assay of varying low (C_t 31-36) medium (C_t 23-28) and high (C_t 17-21) concentrations were tested in 9 replicate reactions in the same run. The results show excellent reproducibility.

Inter-assay reproducibility: The C_t value of the daily positive control was analyzed over a period of 5 days on a calibrated ABI 7500. The results show consistent reproducibility over the period of time on the same sample with mean amplification efficiencies and r^2 values of 97% and 0.997, respectively.

Table G12. Detection of norovirus GI in the presence of IAC and GII

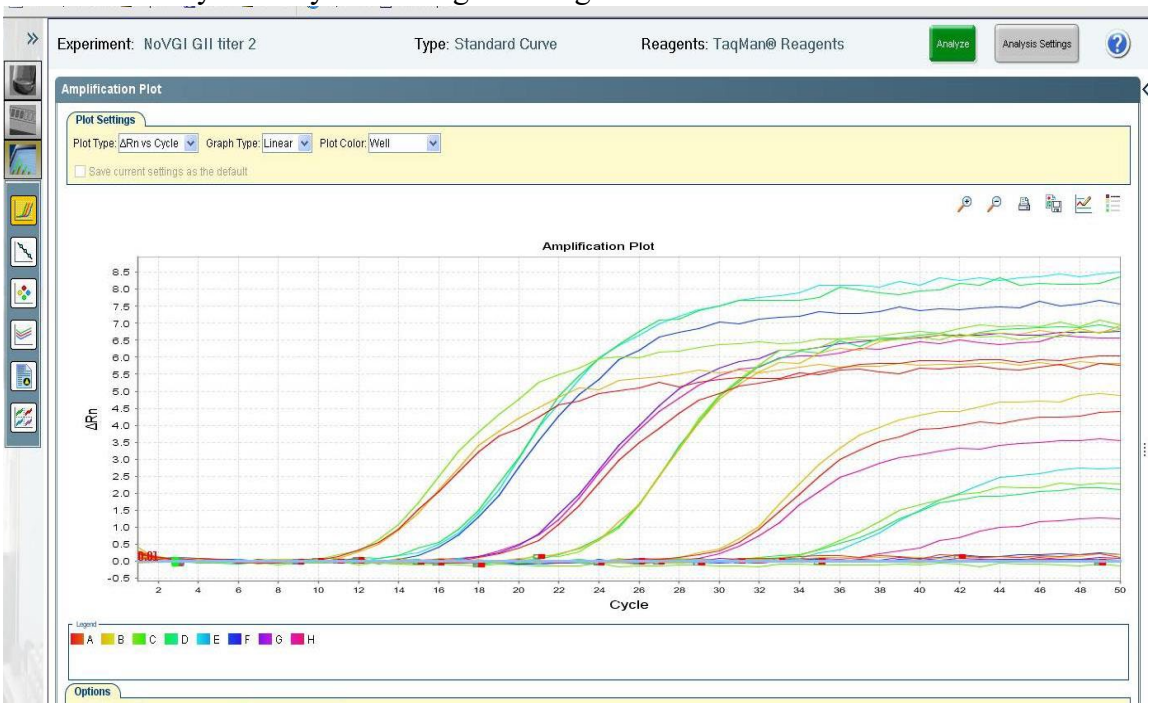
		Without IAC		With IAC			
		GI Average C_t	**GI SD**	**IAC Average C_t**	**IAC SD**	**GI Average C_t**	**GI SD**
With GII	**GI -2 Dilution**	18.04	0.09	28.66	0.34	18.37	0.34
	GI -3 Dilution	21.57	0.18	28.79	0.08	21.79	0.15
	GI -4 Dilution	25.19	0.15	25.64	0.14	28.73	0.11
Without GII	**GI -2 Dilution**	18.06	0.06	28.62	0.31	18.89	0.21
	GI -3 Dilution	21.50	0.09	28.54	0.40	21.73	0.12
	GI -4 Dilution	24.84	0.14	28.63	0.13	24.94	0.08

Table G13. Detection of norovirus GII in the presence of IAC and GI

		Without IAC		With IAC			
		GII Average C_t	**GII SD**	**IAC Average C_t**	**IAC SD**	**GII Average C_t**	**GII SD**
With GI	**GII -2 Dilution**	22.34	0.25	28.55	0.28	22.46	0.28
	GII -3 Dilution	26.30	0.18	28.92	0.23	26.43	0.24
	GII -4 Dilution	29.44	0.39	29.04	0.11	29.83	0.56
Without GI	**GII -2 Dilution**	22.28	0.13	28.76	0.14	22.80	0.28
	GII -3 Dilution	26.05	0.26	28.95	0.06	26.31	0.22
	GII -4 Dilution	29.71	0.53	29.10	0.43	29.41	1.60

Figure G3 (A-B). Dynamic range of the assay.

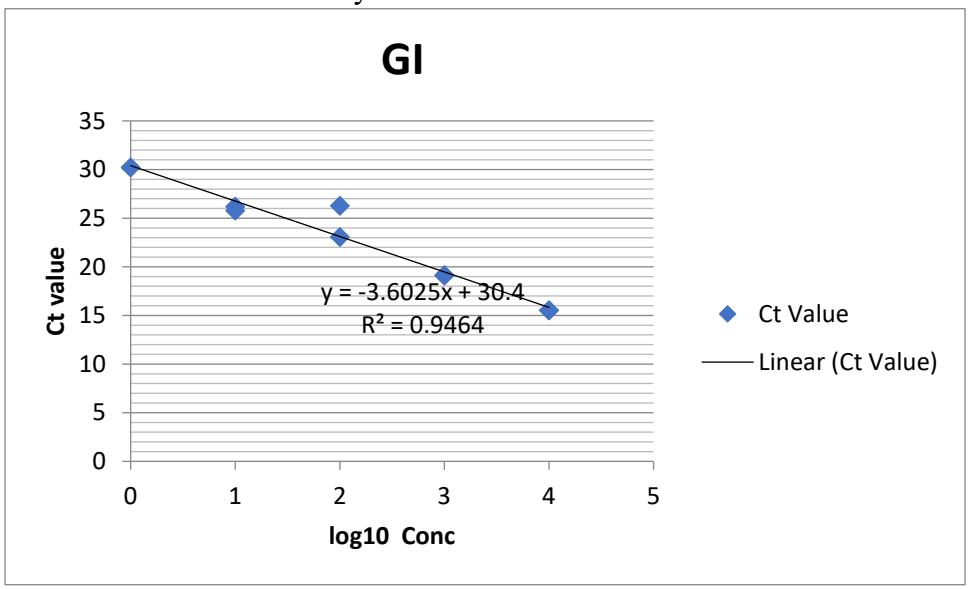
G3 A. The assay has a dynamic range of 7 logs for GI



G3 B. The assay has a dynamic range of 6 logs for GII

Figure G4A-B. Standard curves

G4 A. GI reaction efficiency of 90%



G4 B. GII reaction efficiency of 107%

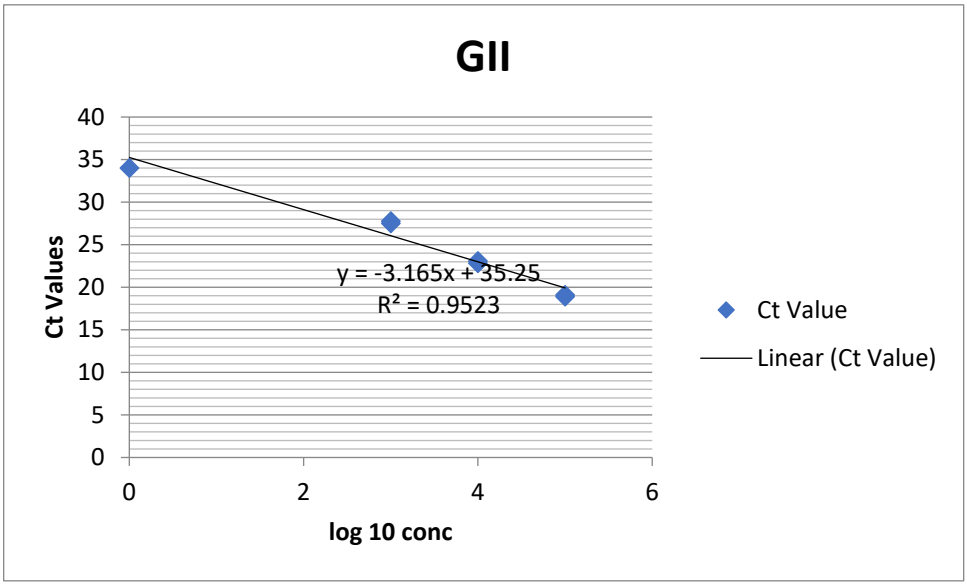


Table G13. LOD/LOQ using GI.1 and GII.4

	Norovirus GI			Norovirus GII		
Genomic copies/rxn	**Average C_t**	**SD***	**Pos/Total**	**Average C_t**	**SD***	**Pos/Total**
100	37.55	0.64	6/6	31.68	0.62	6/6
10	42.79	0.91	6/6	35.06	0.37	6/6
1	47.25	2.37	2/6	41.30	4.13	4/6

*standard deviation of positive samples only.
LOQ= 10 genomic copies
LOD= 1 genomic copies

Multi Laboratory Validation of Norovirus RT-qPCR Detection Assay

The norovirus RT-qPCR detection assay was multi-laboratory validated yielding acceptable results for a Collaborative Validation Study based on FDA Guidelines for the Validation of Analytical Methods for the Detection of Microbial Pathogens in Foods, 2nd Ed (2015). There were 24 enteric organisms included in the MLV for the norovirus multiplex detection assay, with some of the inclusivity samples containing multiple genogroups and strains. There was 100% accuracy and specificity for the detection of norovirus GI while norovirus GII had 99.7% accuracy and 100% specificity-based data from 13 labs (Tables G14 and G15). As indicated in Table G17, there was no inhibition observed in the detection assay.

Table G14. Multi-laboratory validation data for norovirus multiplex detection assay in 3 µl triplicate reaction of inclusivity (norovirus GI and/or GII) or exclusivity (bacteria and other non-human noroviruses) microorganisms[#]

	Strain	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7S	Lab 8	Lab 9	Lab 7A	Lab 11	Lab 12	Lab 13	Lab 14*
1	**GII.4 Minerva**	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3	2 of 3[b]	3 of 3	6 of 3
2	**GI.3B/GII.4 Grim**	6 of 6	6 of 6	6 of 6	6 of 6	6 of 6	6 of 6	6 of 6	6 of 6	6 of 6	6 of 6	6 of 6	6 of 6	6 of 6	6 of 6
3	**GII.12**	3 of 3	4 of 3[a]	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3	5 of 3[a]	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3
4	**GII.12**	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3	4 of 3[a]	4 of 3[a]	6 of 3
5	**GI.3C/GII.16**	6 of 6	6 of 6	6 of 6	6 of 6	6 of 6	6 of 6	6 of 6	6 of 6	6 of 6	6 of 6	6 of 6	6 of 6	6 of 6	6 of 6
6	**GI.4/GII.4∞ Minerva**	5 of 6	6 of 6	6 of 6	6 of 6	6 of 6	5 of 6	6 of 6	5 of 6	3 of 6	6 of 6	6 of 6	6 of 6	6 of 6	3 of 6
7	**GII.2**	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3
8	**GI.6A/GII.4 Osaka**	6 of 6	6 of 6	6 of 6	6 of 6	6 of 6	6 of 6	6 of 6	6 of 6	6 of 6	6 of 6	6 of 6	6 of 6	6 of 6	6 of 6
9	**GII.4 Henry**	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3	2 of 2	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3
10	**GII.1**	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3
11	**GII.4 New Orleans**	3 of 3	3 of 3	4 of 3[a]	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3	0 of 3
12	**GII.6A**	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3	0 of 3
13	**Murine norovirus**	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6
14	**SMSV-17**	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6
15	**Adenovirus 41**	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6
16	**Sabine poliovirus**	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6
17	***Shigella sonnei***	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6
18	**Enterovirus 90**	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6
19	***Vibrio parahaemolyticus***	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6
20	**Vibrio spp.**	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6
21	***Salmonella enterica***	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6
22	**Rotavirus**	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6
23	**HAV HM175f**	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6
24	**Coxsackievirus A1**	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6	0 of 6
25	**Neg Control**	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
26	**GI/GII Pos Control^**	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos

Inclusivity GI: 100% accuracy and 100% specificity of 156 reactions
Inclusivity GII: 99.7% accuracy and 100% specificity of 468 reactions
Exclusivity GI and GII: 100% accuracy
∞For strain GII.4 Minerva – levels were found to be at limit of detection for norovirus GII, therefore detection produced sporadic positive or negatives.
[#] Consists of up to 4,000 C_t values for GI, GII, or IAC
*Excluded data set – analysis performed on a non-calibrated instrument.
[a] Clinical samples 3 and 4 were originally characterized as GII only; however, testing of multiple replicates demonstrated low levels of GI
Reported results of greater than 3 of 3 positive indicates GII was detected in all 3 replicates and GI was detected in additional replicate(s).
[b] False negative GII
^GI.1 and GII.4 RNA transcripts were used as positive control

Table G15. Summary of detection: inclusivity and exclusivity from 13 labs

Organism/Strain	Accuracy	False Negatives	False Positives
GII.4 Minerva	99.7%	0.3%	-
GI.3B	100%	0%	-
GII.4 Grimsby	100%	0%	-
GII.12*	100%	0%	-
GII.12*	100%	0%	-
GI.3C	100%	0%	-
GII.16	100%	0%	-
GI.4	100%	0%	-
GII.4 Minerva	100%	0%	-
GII.2	100%	0%	-
GI.6A	100%	0%	-
GII.4 Osaka	100%	0%	-
GII.4 Henry	100%	0%	-
GII.1	100%	0%	
GII.4 New Orleans*	100%	0%	-
GII.6A	100%	0%	-
Murine norovirus	100%	-	0%
SMSV-17	100%	-	0%
Adenovirus 41	100%	-	0%
Sabine poliovirus	100%	-	0%
Shigella sonnei	100%	-	0%
Enterovirus 90	100%	-	0%
Vibrio parahaemolyticus	100%	-	0%
Vibrio spp.	100%	-	0%
Salmonella enterica	100%	-	0%
Rotavirus	100%	-	0%

* Low-level GI from clinical samples

Table G16. Inter-laboratory repeatability for norovirus RT-qPCR from 13 labs

Norovirus Strain	MEAN*	SD	SE
GII.4 Minerva	27.20	0.59	0.34
GI.3B	34.05	0.28	0.16
GII.4 Grimsby	37.24	0.57	0.33
GII.12	18.45	0.83	0.48
GII.12	25.99	1.13	0.65
GI.3C	25.34	0.54	0.31
GII.16	26.25	0.51	0.29
GI.4	27.78	0.65	0.38
GII.4 Minerva	39.80	1.13	0.73
GII.2	26.33	0.47	0.27
GI.6A	36.82	0.38	0.22
GII.4 Osaka	38.50	0.56	0.32
GII.4 Henry	27.18	0.37	0.21
GII.1	19.73	1.29	0.74
GII.4 New Orleans	30.89	1.14	0.66
GII.6A	21.55	0.46	0.27

* Mean C_t value for all RT-qPCR reactions for each laboratory detection of norovirus strains

Table G17. IAC Inter-laboratory repeatability for IAC from 13 labs

Norovirus Strain	MEAN*	SD	SE
GII.4 Minerva	23.24	1.03	0.15
GI.3B	23.64	0.96	0.14
GII.4 Grimsby	23.64	0.96	0.14
GII.12	24.28	1.17	017
GII.12	25.18	1.93	0.29
GI.3C	23.70	1.16	0.16
GII.16	23.70	1.16	0.16
GI.4	24.10	1.40	0.21
GII.4 Minerva	24.10	1.40	0.21
GII.2	26.16	1.98	0.30
GI.6A	23.07	0.85	0.13
GII.4 Osaka	23.07	0.85	0.13
GII.4 Henry	23.08	0.92	0.13
GII.1	23.43	0.99	0.15
GII.4 New Orleans	23.38	1.01	0.14
GII.6A	24.14	1.43	0.22

* Mean C_t value for all RT-qPCR reactions for each laboratory detection of internal amplification control (IAC). These results indicate minimal inhibition.

Conclusion

The results of the validations for the RT-qPCR detection of norovirus GI and GII are sensitive, specific, reproducible and robust. Our conclusion is that this assay can be used for the detection of norovirus in RNA preparations obtained from any food matrix. This assay is ready to be incorporated into the Bacteriological Analytical Manual and ongoing Office of Regulatory Affairs Field Assignments.

Data Analysis for Norovirus GI and GII Detection Assay

For this norovirus 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. The negative RT-qPCR control sample demonstrates positive results crossing the Cy5 or Cy3 threshold or if the IAC is negative, repeat the RT-qPCR assay,
    b. The RT-qPCR positive control is negative for GI and/or GII,
    c. The average of the IAC C_t values for the sample replicates are more than 4.0 C_ts greater than the Negative Control IAC C_t value, repeat the RT-qPCR assay using remaining RNA or RNA from a newly extracted saved tube with a 1 µl RT-qPCR reaction in triplicate. If the 1 µl template reactions yield an average IAC Ct value greater than 4.0 Ct higher than the Negative Control IAC Ct value, repeat the sample analysis from the beginning using additional food sample. With the new sample, the concentrates will be split into 5 tubes and complete RT-qPCR with 1 µl reactions in triplicate.

Note: A positive sample is a result that demonstrates log amplification. Log amplification can be viewed as a graph on the Smart Cycler and AB 7500 platforms. If the sample does not exhibit

log amplification and crosses the threshold, the RT-qPCR reaction should be repeated. Refer to figures in Appendix E for appropriate amplification curves.

Supplemental Material for Norovirus GI and GII Detection Assay

Table G6. AB7500 HAV, MNV, and Norovirus Buffer Mix Setup*

Reagents	**Volume (50 mM MgCl)**	**Volume (25 mM MgCl)**
DNase/Rnase free H_2O	1760 µl	1610 µl
5X Buffer	1000 µl	1000 µl
MgCl	150 µl	30 µl
dNTPs	200 µl	200 µl

* Made with components from Qiagen One-Step RT-qPCR kit and PCR grade water

Table G7. Enzyme Mix Setup

Component	**Volume**
One-Step Qiagen enzyme	~200 µL
Superase-IN (10000U)	50 µL

Table G8. Norovirus Primer Mix Setup*

Primers/H_2O	**Volume**
COG1F	30 µl
COG1R	30 µl
COG2F	30 µl
COG2R	30 µl
IC46	7.44 µl
IC194	7.44 µl
Dnase/Rnase water	665.12 µl

* Made with 100 µM primers stocks and PCR grade water

Table G9. Norovirus Probe Mix*

Primers/H_2O	**Volume**
G2P	10 µl
GIP	10 µl
GIPb	10 µl
ICP	15 µl
Dnase/Rnase water	355 µl

* Made with 100 µM probe stocks and PCR grade water

Appendix H: Data Analysis and Supplemental Material for the Mengovirus Detection Assay

The method detection assay described here was developed by CFSAN's Gulf Coast Seafood Laboratory for use as an alternative extraction control (to murine norovirus). This is an RT-qPCR assay for the detection of Mengovirus with the inclusion of an internal amplification control (IAC). All primers and probes should be hydrated to a concentration of 100 µM using primer TE prior to making primer and probe mixes.

Data Analysis for the Menogovirus Detection Assay

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

1. Repeat all invalid samples, a sample is "invalid" if:

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

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

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

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

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

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

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

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

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

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

qPCR reactions due to inhibition is to ensure that you do not have false negatives. Refer to figures in Appendix E for appropriate amplification curves.

Supplemental Material for the Mengovirus Detection Assay

Table H1. Mengovirus Buffer Mix Setup*

Reagents	**Volume (50 mM MgCl)**	**Volume (25 mM MgCl)**
DNase/RNase free H_2O	1760 µl	1610 µl
5X Buffer	1000 µl	1000 µl
$MgCl_2$	150 µl	300 µl
dNTPs	200 µl	200 µl

* Made with components from Qiagen One-Step RT-qPCR kit and PCR grade water

Table H2. Enzyme Mix Setup

Component	**Volume**
One-Step Qiagen enzyme	~200 µL
Superase-IN (10000U)	50 µL

Table H3. Mengovirus Primer Mix Setup*

Primers/H_2O	**Volume**
MengoF	15 µl
MengoR	15 µl
IC46F	5.58 µl
IC194R	5.58 µl
DNase/RNase water	558.84 µl

* Made with 100 µM primers stocks and PCR grade water

Table H4. Mengovirus Probe Mix

Probe/H_2O	**Volume**
MengoP	18.75 µl
ICP	11.5 µl
DNase/RNase water	270.01 µl

* Made with 100 µM probe stocks and PCR grade water

Appendix I: Murine Norovirus, Hepatitis A Virus, Norovirus Genogroup I, and Norovirus Genogroup II Detection Assays on the Smart Cycler

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

RT-qPCR Detection of Murine Norovirus on Smart Cycler Platforms

RT-qPCR Assays

Outlined Murine Norovirus RT-qPCR for detection of murine norovirus on Smart Cycler. Primers, probes, and master mix preparation are found in Tables I1 and I2.

Murine Norovirus Protocol

Reverse transcription: 50 °C for 3000 sec

Activation: 95 °C for 900 sec

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

Murine Norovirus Reaction Set-Up Smart Cycler

Note: Always use aerosol resistant pipette tips for PCR.

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

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

3. Prepare master mix for all sample and control reactions as listed in table 2. Keep all thawed components, reagents, controls, and master mixes **in cooling block or on ice**.

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

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

5. Add 22 µl master mix to each designated reaction tube or sample wells.

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

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

Instrument Set-Up Smart Cycler

1. Place reactions tubes in the Smart Cycler and create run. Make sure the appropriate dye set (FCTC25) and protocols are selected for each site.

2. Start run; the entire reaction time for this assay is approximately 3 hrs.

Data Analysis Smart Cycler

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

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

3. Usage: Assay

4. Curve Analysis: Primary

5. Threshold Setting: Manual

6. Manual Threshold Fluorescence Units: 10.0

7. Auto Min Cycle: 5

8. Auto Max Cycle: 10

9. Valid Min Cycle: 3

10. Valid Max. Cycle: 60

11. Background subtraction: ON

12. Boxcar Avg. Cycles: 0

13. Background Min. Cycle: 5

14. Background Max. Cycle: 40

15. Max Cycles: 45

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

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

Murine Norovirus Data Analysis

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

    a. The RT-qPCR negative control demonstrates positive C_t results for MNV in Cy5 or if the IAC is negative (no C_t from TxRed),

    b. The RT-qPCR positive control is negative (no C_t from Cy5) for MNV,

    c. The MNV RT-qPCR is negative (no C_t from Cy5) for any sample,

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

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

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

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

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

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

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

Table I1. Primer and Probe Sequences for MNV and Internal Amplification Control

Identification	Primers	Location[#]
MNVR[@]	5' CAC AGA GGC CAA TTG GTA AA 3'	6645-6626
MNVF	5'- TGC AAG CTC TAC AAC GAA GG -3'	6520-6539
IC46F[a]	5'- GAC ATC GAT ATG GGT GCC G-3'	N/A
IC194R[a]	5'- AAT ATT CGC GAG ACG ATG CAG -3'	N/A
MNVP	Cy5- 5' CCT TCC CGA CCG ATG GCA TC3'-IB-RQ*	6578-6594
IACP	TxR – 5' TCT CAT GCG TCT CCC TGG TGA ATG TG -IB RQ 3' *	N/A

[@] Hewitt, Rivera-Aban, Greening 2009
[a] Depaola, Jones, Woods et. al. 2010 Internal Amplification Control (IAC) primers and probes are covered by U.S. Patent Application 0060166232.
* IB RQ- Iowa Black RQ
[#] Based on accession no. JF320650

Table I2. Smart Cycler Amplification Reaction Components and Master Mix Volume for MNV

Reagent	Initial Concentration	Volume per 25 µl reaction	Final Concentration
RNase Free H_20		11.8 µl	-
5X OneStep RT-PCR Buffer	5X	5.0 µl	1X
$MgCl_2$ ~	50 mM	0.75 µl	1.5 mM
dNTP Mix	10 mM	1 µl	0.4 mM
MNVF	10 µM	0.50 µl	0.2 µM
MNVR	10 µM	0.50 µl	0.2 µM
IC 46F	10 µM	0.1875 µl	0.075 µM
IC 194R	10 µM	0.1875 µl	0.075 µM
MNVP	10 µM	0.25 µl	0.1 µM
IACP	10 µM	0.375 µl	0.15 µM
OneStep RT-PCR Enzyme Mix		1.00 µl	
Superase·in	20 Units/µl	0.25 µl	5 Units
Internal Amplification Control RNA		*0.2 µl	-
RNA		3 µl	

* Amount varies with concentration of IAC RNA. The amount of IAC template needs to be adjusted based on the prepared stock concentration to report Cycle threshold (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 $MgCl_2$, the final concentration per reaction is 4.0 mM $MgCl_2$

RT-qPCR Detection of Hepatitis A Virus Smart Cycler Platform

RT-qPCR Assay

Outlined Hepatitis A Virus RT-qPCR for detection of hepatitis A on Smart Cycler. Primers, probes, and master mix preparation are found in Tables I3-4.

Hepatitis A Virus Protocol

Reverse transcription: 50 °C for 3000 sec

Activation: 95 °C for 900 sec

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

Hepatitis A Virus Reaction Set-Up Smart Cycler

Note: Always use aerosol resistant pipette tips for PCR.

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

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

3. Prepare master mix for all sample and control reactions as listed in table 6. Keep all thawed components, reagents, controls, and master mixes **in cooling block or on ice**.

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

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

5. Add 22 µl master mix to each designated reaction tube or sample wells.

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

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

Instrument Set-Up Smart Cycler

1. 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, and analysts initials.

2. Start run; the entire reaction time for this assay is approximately 3 hrs.

Data Analysis Smart Cycler

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

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

3. Usage: Assay

4. Curve Analysis: Primary

5. Threshold Setting: Manual

6. Manual Threshold Fluorescence Units: 10.0

7. Auto Min Cycle: 5

8. Auto Max Cycle: 10

9. Valid Min Cycle: 3

10. Valid Max. Cycle: 60

11. Background subtraction: ON

12. Boxcar Avg. Cycles: 0

13. Background Min. Cycle: 5

14. Background Max. Cycle: 40

15. Max Cycles: 50

16. Any sample which crosses the threshold in the Cy5 (Ch. 4) channel will be demonstrate detection of HAV.

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

Table I3. Primer and Probe Sequences for HAV and Internal Amplification Control RNA

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

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

Table I4. Smart Cycler Amplification Reaction Components and Master Mix Volume for HAV

Reagent	**Initial Concentration**	**Volume per 25 µl reaction**	**Final Concentration**
RNase Free H_20		11.05 µl	-
5X OneStep RT-PCR Buffer	5X	5.0 µl	1X
$MgCl_2$[a]	50 mM	0.75 µl	1.5 mM
dNTP Mix	10 mM	1 µl	0.4 mM
GAR2F	10 µM	0.75 µl	0.3 µM
GAR1R	10 µM	0.75 µl	0.3 µM
IC 46F	10 µM	0.1875 µl	0.075 µM
IC 194R	10 µM	0.1875 µl	0.075 µM
GARP	10 µM	0.5 µl	0.2 µM
IACP	10 µM	0.375 µl	0.15 µM
OneStep RT-PCR Enzyme Mix		1.00 µl	
Superase·in	20 Units/µl	0.25 µl	5 Units
Internal Amplification Control RNA		[b]0.2 µl	-
RNA		3 µl	

[a]With the addition of 1.5 mM MgCl, the final concentration per reaction is 4.0 mM MgCl.
[b]Amount varies with concentration of IAC RNA. The amount of IAC template needs to be adjusted based on the prepared stock concentration to report Cycle threshold (C_t) of 20-25 when no inhibition is present in the reaction.

RT-qPCR Detection of Norovirus GI and GII on Smart Cycler Platforms

RT-qPCR Assay

Outlined norovirus RT-qPCR for detection norovirus GI and GII on Smart Cycler. Primers, probes, and master mix preparation are found in Tables I5-6.

Norovirus Virus Protocol

Reverse transcription: 50 °C for 3000 sec

Activation: 95 °C for 900 sec

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

Reaction Set-Up Smart Cycler

Note: Always use aerosol resistant pipette tips for PCR.

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

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

3. Prepare master mix for all sample and control reactions as in Appendix F. Keep all thawed components, reagents, controls, and master mixes **in cooling block**.

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

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

8. Add 22 µl master mix to each designated reaction tube or sample wells.

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

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

Instrument Set-Up Smart Cycler

1. 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, and analysts initials.

2. Start run; the entire reaction time for this assay is approximately 3 hrs.

Data Analysis Smart Cycler

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

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

3. Usage: Assay

4. Curve Analysis: Primary

5. Threshold Setting: Manual

6. Manual Threshold Fluorescence Units: 10.0

7. Auto Min Cycle: 5

8. Auto Max Cycle: 10

9. Valid Min Cycle: 3

10. Valid Max. Cycle: 60

11. Background subtraction: ON

12. Boxcar Avg. Cycles: 0

13. Background Min. Cycle: 5

14. Background Max. Cycle: 40

15. Max Cycles: 50

16. Any sample which crosses the threshold in the Cy5 (Ch. 4) channel will be demonstrate detection of norovirus GI, any sample which crosses the threshold in the Cy3 (Ch. 2) will demonstrate detection of norovirus GII.

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

Table I5. Primer and Probe Sequences for Norovirus and Internal Amplification Control RNA

Identification	**Primers**	**Location**
COG1R[a,d]	5’ CTT AGA CGC CAT CAT CAT TYA C 3’	5350-5371
COG2R[a,e]	5’ TCG ACG CCA TCT TCA TTC ACA 3’	5080-5100
COG1F[a,d]	5’ CGY TGG ATG CGN TTY CAT GA 3’	5287-5306
COG2F[a,e]	5’ CAR GAR BCN ATG TTY AGR TGG ATG AG 3’	5003-5028
IC46F[b,c]	5’GAC ATC GAT ATG GGT GCC G-3’	N/A
IC194R[b,c]	5’-AAT ATT CGC GAG ACG ATG CAG-3’	N/A
	Probes	
COGP[a,d]	Cy5- 5’ (TAO) AGA TYG CGA TCY CCT GTC CA 3’ -IB-RQ*	5317-5336
COGP1b[a,d]	Cy5- 5’ (TAO) AGA TCG CGG TCT CCT GTC CA 3’ -IB-RQ*	5317-5336
COG2P[a,e]	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.
[d] Based on GenBank accession # KF039728
[e] Based on GenBank accession # EF684915
* IB RQ- Iowa Black RQ

Table I6. Smart Cycler Amplification Reaction Components for Norovirus

Reagent	**Initial Concentration**	**Volume per 25 µl reaction**	**Final Concentration**
RNase Free H_20		9.3 µl	-
5X OneStep RT-PCR Buffer	5X	5.0 µl	1X
$MgCl_2$[a]	50 mM	0.75 µl	1.5 mM
dNTP Mix	10 mM	1 µl	0.4 mM
COG1F	10 µM	0.75 µl	0.3 µM
COG1R	10 µM	0.75 µl	0.3 µM
COG2F	10 µM	0.75 µl	0.3 µM
COG2R	10 µM	0.75 µl	0.3 µM
IC 46F	10 µM	0.1875 µl	0.075 µM
IC 194R	10 µM	0.1875 µl	0.075 µM
COG1P	10 µM	0.25 µl	0.1 µM
COG1Pb	10 µM	0.25 µl	0.1 µM
COG2P	10 µM	0.25 µl	0.1 µM
IACP	10 µM	0.375 µl	0.15 µM
OneStep RT-PCR Enzyme Mix		1.00 µl	
Superase·in	20 Units/µl	0.25 µl	5 Units
Internal Amplification Control RNA		[b]0.2 µl	-
RNA		3 µl	

[a] With the addition of 1.5 mM $MgCl_2$, the final concentration per reaction is 4.0 mM $MgCl_2$

[b] 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.

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