Performing the GI Virus Panel by Real-Time PCR Procedure

Purpose: This procedure provides instructions for how to perform the GI Virus Panel by Real-Time Polymerase Chain Reaction (q-PCR) to detect Sapovirus(Sapo), Rotavirus(Rota), Adenovirus(Adv) and Astrovirus(Ast) from patient's fecal and vomitus samples using a standardized methodology.

I. Samples: Nucleic acid from patient fecal and vomitus samples extracted using the bioMérieux easyMAG. See PHGI_101_1600 for acceptable sample types and collection containers.

II. Materials:

A. Media: N/A

B. Primers and Probes

Target	Primer	Sequence (5'- 3')	Genomic Target	Manufacturer
Sapovirus	Sav1F	TTG GCC CTC GCC ACC TAC	Junction of polymerase and capsid	Life Technologies
	Sav5F	TTT GAA CAA GCT GTG GCA TGC TAC		
	Sav124F	GAY CAS GCT CTC GCY ACC TAC		
	Sav124R	CCC TCC ATY TCA AAC ACT A		
Rotavirus Type A	NSP3-F	ACC ATC TWC ACR TRA CCC TCT ATG AG	Non structure Protein 3	Life Technologies
	NSP3-R	GGT CAC ATA ACG CCC CTA TAG C		
Adenovirus 40/41	AdV-F	GCC TGG GGA ACA AGT TCA G	Hexon	Life Technologies
	AdV-R	ACG GCC AGC GTA AAG CG		
Astrovirus	Ast-F	AAG CAG CTT CGT GAR TCT GG	Junction of polymerase and capsid	Life Technologies
	Ast-R	GCC ATC RCA CTT CTT TGG TCC		

Target	*Probe*	*Sequence(5'- 3')*	*Reporter*	*Quencher*	Manufacturer
Sapovirus	Sav124TP	CCR CCT ATR AAC CA	FAM	MGB-NFQ	Life Technologies
	Sav5TP	TGC CAC CAA TGT ACC A			
Rotavirus Type A	NSP3-P	AGT TAA AAG CTA ACA CTG TCA AA	VIC	MGB-NFQ	Life Technologies
Adenovirus 40/41	AdV-P	ACC CAC GAT GTA ACC AC	NED	MGB-NFQ	Life Technologies
Astrovirus	Ast-P	CAC AGA AGA GCA ACT CCA TCG CAT TTG	Cy5	Tao-IBDRQ	Integrated DNA Technologies

C. Other Reagents, Supplies and Equipment:

Reagent	Supplies	Equipment
Applied Biosystems TaqMan® Fast Virus 1-Step Master Mix(cat# 4444434)	Applied Biosystems Fast Optical 96-Well Plates or MicroAmp Optical 8-well strips	ABI 7500 Real-Time PCR System with Fast Block

IDTE 1x TE Buffer pH8.0	MicroAmp Optical Adhesive Film (P/N4311971) and Applicator or MicroAmp Optical 8-well strip caps	Biological safety cabinet- 6ft, Nuaire, Class II Type A/B3
UltraPURE DNAse/RNAse Free water	Sterile Barrier Tips for pipets	Pipettes (2.5 µL, 10 µL, 20 µL, 100 µL and 1000 µL)
DNA AWAY, Molecular BioProducts	Disposable Powder-free Gloves and Gowns	Mini-Micro Vortex Mixer
gBlocks® control oligos (see table C)	Under pad, disposable	96-well plate centrifuge
Custom primers and probes (see table B)		

D. gBlocks Control Oligos (Integrated DNA Technologies)

gBlock	Sequence
Human adenovirus type 40/41 gBlock	5'- GCCTGGGGAACAAGTTCAGAAACCCCACCGTGGCTCCGACCCACG ATGTAACCACAGACAGGTCACAGCGACTGACGCTGCGATTCGTGCC CGTCGACCGCGAGGAAACCGCCTACTCTTACAAAGTGCGCTTTACG CTGGCCGT - 3'
Human astrovirus gBlock	5'- TGGAGCACTGCCTCTCACGGACTGCAAAGCAGCTTCGTGACTCTGG CCTTCCA GCCAGACTCACAGAAGAGCAACTCCATCGCATTTGGAGGGGAGGA CCAAAGAAGTGTGATGGCTAGCAAGTCCAATA - 3'
Human rotavirus type A gBlock	5'- ACTATGAATATGCATATGAGTAGTCACATAATAAAGAATGTTCACCAT CTACACA TGACCCTCTATGAGCACAATAGTTAAAAGCTAACACTGTCAAAAACC TAAATGGCTATAGGGGCGTTATGTGACC - 3'
Human sapovirus Polymerase/Capsid Junction gBlock®	*SaV124* 5'- TAACACCAACTATGACCAGGCTCTCGCCACCTACAATGCTTGGTTCA TAGGTGGTACAGTACCTGA CCCAGTGGGTTACACTGAAGGAACCCACAAAATAGTGTTTGAGATG GAGGGCAATGGCTCCAAC - 3'
	SaV1 5'- TGAATACAAATTTTGATTTGGCCCTCGCCACCTACAATGCCTGGTTC ATAGGTGGTACAGCTCCA GATCCAGAGCGCCCCACTGAAGGTGCACCCAAATTAGTGTTTGAGA TGGAGGGCAATGGCTCCA - 3'
	SaV5 5'- GTTAATACCAACTTTGAACAAGCTGTGGCATGCTACAACAGCTGGTA CATTGGTGGCACCACACC AGAAATGCCCACTACCAATGAAGGCTGTGGGCTATTAGTGTTTGAG ATGGAGGGCAATGGCTCCC - 3'

III. Quality Control:

A. Positive control

IDT gBlocks® or suitable extracts of known positive targets diluted such that the Ct value is between 29 and 31 cycles when the threshold is set at a ΔRn value of 0.05. Currently the controls are set to be:

Astrovirus gBlock	12500 copies per reaction
Adenovirus gBlock	1000 copies per reaction
Rotavirus gBlock	1000 copies per reaction
Sapovirus RNA Extract	Aim for CT value between 27-31

B. Negative (no template control -NTC)

The negative PCR control is DEPC water substituted for sample.

C. Extraction control (Mean ± 2SD)

DEPC water follows the entire extraction protocol and should be used as an extraction control.

D. Inhibition control

All fecal extracted and first tested for Norovirus which includes an exogenous internal control West Nile Virus. All samples should pass the detection of -WNV before carrying on to the GI panel test.

WNV should be detected in all samples . Any sample that is negative for WNV is either inhibited or insufficient and must be repeated.

IV. Procedure:

A. Running the q-PCR on the TaqMan 7500

	Action	Related Documents Title Number
	Note: *This assay was specifically designed to detect Human sapovirus, Human rotavirus type A, Enteric adenovirus types 40/41 and Human astrovirus Types 1-8 all associated with gastroenteritis. The assay has not been designed or validated to detect other types that cause infection in other body sites.*	BCCDC Laboratory Safety Manual
1.	Upload sample extraction file into the Norofast run template on the ABI 7500 Fast Real-Time PCR System.	
2.	Turn on the BSCs in the PCR reagent clean room and in the PCR genomic extraction clean room, assure stable functionality and wipe down all surfaces inside the BSC with DNA Away prior to starting work and record QC.	BCCDC Safety Manual, Section F-BSCs
3.	Thaw all qPCR cocktail components in the refrigerator cold block. ***Note****: The 20x primer/probe mix should be prepared ahead of time and QC'd before use in a sample run. See Appendix A for recipe.*	
4.	Set-up the GI panel qPCR cocktail using the TaqMan® Fast Virus 1-Step Mix q-PCR Kit by multiplying the 1x recipe by the number of samples and controls to be run, plus approximately 10% overage. **Reagent** / **Vol./reaction (µl)** PCR grade Water — 9 TaqMan® Fast Virus 1-Step Mix (4X) — 5 20x GI panel multiplex primer/probe mix — 1 ***Note:*** *Record the Lot Numbers for all PCR Master Mix reagents in the spaces provided on the worksheet.*	
5.	Remove an Applied Biosystems Fast Optical 96-Well Plate and or MicroAmp Optical 8-well strip from the box, inside the BSC, and place it in a MicroAmp 96-Well Plate Holder to ensure that the bottom of the plate has no contact with anything that could fluoresce. ***Note:*** *<u>Do not</u> use permanent marker on optical plates, the ink will fluoresce.*	
6.	Aliquot 15µL of the GI panel cocktail to the wells being used for the assay in the plate/strips. ***Note:*** *Only use ABI Fast Optical 96-Well Reaction Plates or MicroAmp Optical 8-well strips in the fast block enabled TaqMan 7500.*	
7.	Transport the qPCR plate/strips in a ziploc bag to the genomic preparation room. Do not remove the plate from the bag until you are ready to add the sample extracts.	
8.	Place the qPCR plate/strips in the plate holder in BSC.	
9.	Add 5µL of control or sample to the appropriate well of the 96-well plate/strip as specified by the extraction/plate layout sheet from the 7500.	

<table>
<tr><th></th><th>Action</th><th>Related Documents Title Number</th></tr>
<tr><td></td><td>Note: This assay was specifically designed to detect Human sapovirus, Human rotavirus type A, Enteric adenovirus types 40/41 and Human astrovirus Types 1-8 all associated with gastroenteritis. The assay has not been designed or validated to detect other types that cause infection in other body sites.</td><td>BCCDC Laboratory Safety Manual</td></tr>
<tr><td>10.</td><td>Seal the plate with a MicroAmp optical plate film or cap the optical 8-well strips with the MicroAmp optical 8-well strip caps using the plate film applicator.

Note: Be careful not contaminate the plate film optical surface with anything that could fluoresce such as ink, dust or fingerprints.</td><td></td></tr>
<tr><td>11.</td><td>Gently tap the plate/strips to remove air bubbles from the sides and bottom of each well. If the bubbles are still present, spin the plate/strips down in a plate centrifuge.

Note: Place the plate/strips back in the zip-loc bag if it needs to be spun down so it is not contaminated with dust. Do not run a plate with reaction droplets on the underside of the optical film, spin down the plate if this occurs.</td><td></td></tr>
<tr><td>12.</td><td>Place the sealed plate/strips back in the zip-loc bag to transport it to the ABI 7500.

Note: transporting the plate/strips in a zip-loc bag prevents contamination with dust etc. prior to loading on the instrument.</td><td></td></tr>
<tr><td>13.</td><td>Load the plate/strips into the Fast Block enabled ABI 7500.

Note: Use the appropriate sample loading rack on the Fast block.</td><td>PHGI_283_2500 Using the 7500 Fast Real-Time PCR System</td></tr>
<tr><td>14.</td><td>Amplify the samples on the ABI Fast 7500 using the following program stored as GI PANEL template with the fast qPCR protocol:

Fast Run Protocol:

<table>
<tr><th>Step</th><th>Temperature (°C)</th><th>Time (min:sec)</th><th>cycles</th></tr>
<tr><td>UNG activation* (or RT)</td><td>50</td><td>5:00</td><td>1</td></tr>
<tr><td>Taq activation</td><td>95</td><td>0:20</td><td>1</td></tr>
<tr><td rowspan="2">Amplification</td><td>95</td><td>0:03</td><td rowspan="2">40</td></tr>
<tr><td>60</td><td>0:30</td></tr>
<tr><td colspan="4">*Note: Published UNG activation for TaqMan Fast Advance 2x Master Mix is 2 minutes. Holding the reactions at 50°C for 5 minutes does not adversely affect master mix performance.</td></tr>
</table>

Confirm that the cycling program is correct before starting the instrument and the all detectors are selected for each sample</td><td></td></tr>
<tr><td>15.</td><td>Initiate the run by clicking on the start button on the computer screen.</td><td></td></tr>
<tr><td>16.</td><td>Confirm that the instrument is running before you leave.</td><td></td></tr>
</table>

B. Results Analysis

<table>
<tr><th></th><th>Action</th><th>Related Documents
Title
Number</th></tr>
<tr><td></td><td>Note: Use Universal Precautions and appropriate PPE when handling samples. Discard wastes according to the Waste Management Policy. Processing to be performed in a BSC. All sample handling occurs in the PCR Genomic Extraction Clean Room; all reagent preparation is done in the PCR Reagent Clean Room</td><td>BCCDC Laboratory Safety Manual</td></tr>
<tr><td>1.</td><td>Open the ANALYSIS setting window and ensure that the C_T Threshold for all targets is set to 0.05.</td><td></td></tr>
<tr><td>2.</td><td>Analyze the q-PCR results of the controls for each of the primer sets;
<table>
<tr><th>If</th><th>Then</th></tr>
<tr><td>the CT value for the negative control is Undetermined</td><td>Proceed to analyse the positive controls</td></tr>
<tr><td>the CT value for the negative control is NOT Undetermined</td><td>Check the multicomponent plot
<table>
<tr><th>If</th><th>Then</th></tr>
<tr><td>there is actually a rise in fluorescence relative to the baseline ROX</td><td>• The run has failed due to contamination.
• Consult your supervisor and repeat the run with reagents that have passed QC.</td></tr>
<tr><td>there is No rise in fluorescence relative to the baseline ROX</td><td>• There may be background fluorescence in the wells or the plate.
• Inform your supervisor</td></tr>
</table></td></tr>
</table></td><td></td></tr>
<tr><td>3.</td><td>Check the CT value for all positive controls
<table>
<tr><th>If</th><th>Then</th></tr>
<tr><td>The Positive Control has a C_T value within its quality control range for all targets

NOTE: See QC Stat Tools for details</td><td>The run is valid and the sample results can be analyzed.</td></tr>
<tr><td>The Positive Control does not have a C_T value within its quality control range.</td><td>The positive control may be failing. Repeat the run using the old and a new control.</td></tr>
</table></td><td></td></tr>
</table>

<table>
<tr><td>4.</td><td>Analyze the q-PCR results of the patient samples
<table>
<tr><th>If</th><th>Then</th></tr>
<tr><td>Ct values are not detected for the target or are Undetermined</td><td>No Sapovirus, Rotavirus, Adenovirus, and Astrovirus was detected in the patient sample</td></tr>
<tr><td>The sample has a Ct value > 35 cycles, but < 40 cycles the result is either false positive or weakly positive.

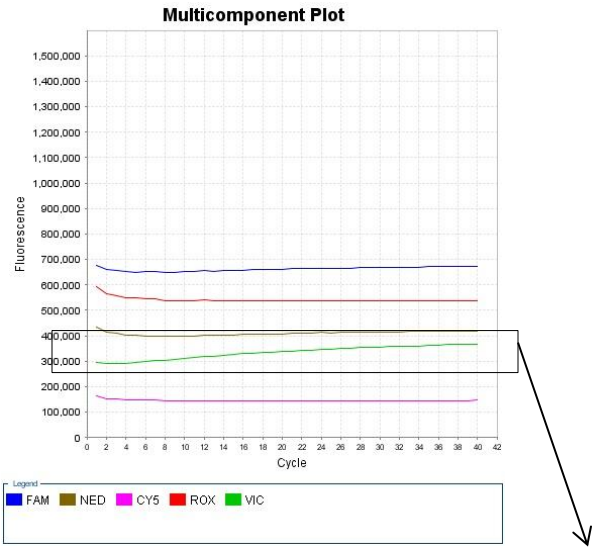
Re-extraction would limit the possibility of it being a contaminated sample during the extraction/loading steps.</td><td>Consult Supervisor. If instructed, repeat testing in duplicate
<table>
<tr><th>If</th><th>Then</th></tr>
<tr><td>A Ct value is reproducible for 2/3 technical replicates</td><td>report as positive for target</td></tr>
<tr><td>A Ct value is not reproducible for 2/3 technical replicates</td><td>Report as negative</td></tr>
<tr><td>Ct values are still inconsistent</td><td>Repeat extraction and run sample in triplicate</td></tr>
</table></td></tr>
<tr><td>The sample has a C_T value less than 35 cycles</td><td>Check the amplification plot for that sample
<table>
<tr><th>If</th><th>Then</th></tr>
<tr><td>The amplification plot forms a normal curve.</td><td>Report the sample as positive for target.</td></tr>
<tr><td>The amplification plot is NOT a normal curve.</td><td>Consult Supervisor and troubleshoot.</td></tr>
</table></td></tr>
</table></td><td></td></tr>
<tr><td>5.</td><td>Export results into GI Panel Workflow Template. Print results and verify the CT values on the printout with the amplification plot and multicomponent plot.</td><td></td></tr>
<tr><td>6.</td><td>Enter control results into the QC Stat Tools and note on worksheet if QC has passed. Pass all worksheets to Supervisor for review.</td><td></td></tr>
<tr><td>7.</td><td>Proceed to PHGI_304_3600 Reporting GIDO Result Entry</td><td></td></tr>
</table>

Procedure Notes:

Method Limitations:

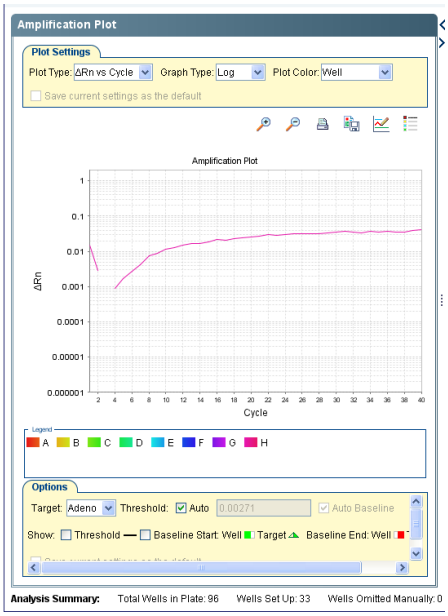
A. **This method has been only validated for fecal and vomitus samples extracted on the bioMérieux EasyMag.**

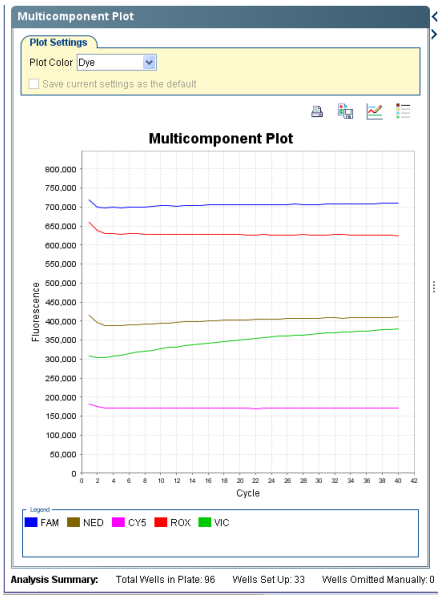
B. **VIC-labelled probe and slope drift.**

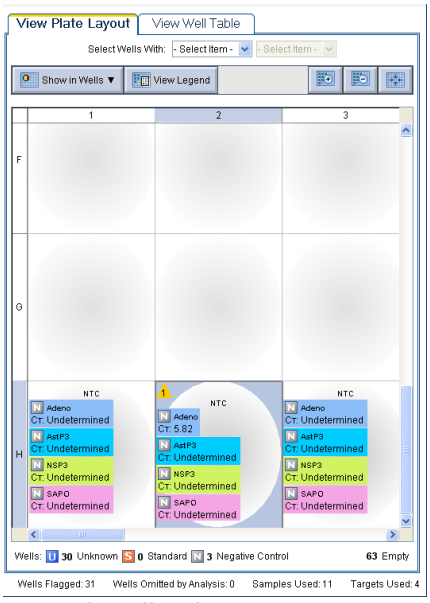


This multicomponent plot exhibits a "signal drift" for the VIC-labelled probe. The reasoning for this is unknown and will occur with a change of dye. Nevertheless, it has been validated against strong positives will not cause false positives. ABI has confirmed that this phenomenon is acceptable.

C. **Poor Analysis by Software:**







Occasionally, the ABI 7500

Occasionally, the software will calculate the math for some samples improperly. As you can see above, the background calculation has produced a positive detection at a Ct value of 5.82(automatic threshold). With a strong Ct value of 5.82 you would expect your multicomponent plot to produce both a strong fluorescence and a normal sigmoidal curve; however upon inspection we can see that there is no evidence of exponential amplification or any amplification for that matter.

There are two options here: you can disregard the amplification based on the multicomponent plot or you can alter the background calculations by manually setting the background cycle count cycles between 3 and 15. Cycle 3 is where actual target length template doubling is occurring and cycle 15 is an approximate value for a typically strong positive that will appear on a plate if the plate contains any positives.

References:

Pang, Xiaoli L., et al. *Journal of Medical Virology: Enhanced Enteric Virus Detection in Sporadic Gastroenteritis using a Multi-Target Real-Time PCR Panel: A One-Year Study*. 86 Vol. John Wiley & Sons Inc, 09/01/2014. Web. 2 Oct. 2015.

Zeng, S. -Q, et al. "One-Step Quantitative RT-PCR for the Detection of Rotavirus in Acute Gastroenteritis." Journal of Virological Methods 153.2 (2008): 238-40. Web.

Feeney, S. A., et al. *Journal of Medical Virology: Development and Clinical Validation of Multiplex TaqMan Assays for Rapid Diagnosis of Viral Gastroenteritis*. 83 Vol. John Wiley & Sons Inc, 09/01/2011. Web. 2 Oct. 2015.

Oka, Tomoichiro, et al. "Detection of Human Sapovirus by real-time Reverse transcription-polymerase Chain Reaction." Journal of Medical Virology 78.10 (2006): 1347-53. Web.

Final Validation Report, *Gastroenteritis Viral Panel Detection using qPCR;* Trevor Hird, MMGP and Environmental Microbiology Laboratory Affiliations, 2016.

Appendix A

Preparation of 20x Primer/Probe mix:

Prepare the GI Panel 20x primer-probe mix used in the qPCR cocktail according to the following recipe:

(Confirm the stock concentration in order to finalize the 20x Mix Recipe)

20x Mix Recipe				
Primer/Probe	[Stock] uM	[20x Mix] uM	[Final] nM	Volume(ul) of stock to add (500ul/rxn final vol.)
Ast-F	200	9	450	**22.5**
Ast-R	200	9	450	**22.5**
Ast-P	100	3	150	**15**
AdV-F	200	8	400	**20**
Adv-R	200	8	400	**20**
Adv-P	100	4	200	**20**
Sav1F	200	4	200	**10**
Sav5F	200	4	200	**10**
Sav124F	200	4	200	**10**
Sav124R	200	4	200	**10**
Sav5TP	100	2	100	**10**
Sav124TP	100	2	100	**10**
NSP3-F	200	6	300	**15**
NSP3-R	200	6	300	**15**
NSP3-P	100	2	100	**10**
			1x IDTE	280
			Total Volume	500

Note: *Store the 20x mix at -20°C in the dark. Excessive exposure to light may affect the fluorescent probes. Do not perform more than 20 freeze-thaw cycles.* ***If you expect to freeze-thaw the 20x mix more than ten times, consider aliquoting to minimize the number of freeze-thaw cycles.***

Revision History

Version	Date	Writer	Description of Change
0.0	July 1, 2016	Trevor Hird	Training Draft
1.0	November 27, 2017	Angela Kong/Stephanie Man	Initial Release

When making reference, use year of latest version.

Protocol developed by Molecular Microbiology & Genomics team at BC CDC.