

Detecting Norovirus by Fast Real-Time RT-PCR

I. Purpose:

This procedure provides instructions for detecting **Norovirus genotypes I (G1) and II (G2)**, by triplex real-time RT-PCR (qRT-PCR) in RNA extracted from stool and vomitus.

Extracted RNA is added to an RT-PCR reaction containing primers and fluorescent probes specific for Norovirus GI, Norovirus GII and Armored West Nile Virus RNA, which acts as an exogenous internal positive control for extraction efficiency and PCR inhibition. The data is analyzed with manual thresholds and cycle thresholds (Ct's) for each target are generated, followed by visual confirmation of instrument-generated results and assignment and reporting of results by the technologist.

This SOP replaces PHGI_203_2003.

II. Samples:

A.

1. RNA extracts from stool or vomitus specimens.

III. Materials:

A. Media

N/A

B. Primers and Probes

Target	Primer	Sequence (5'- 3')	Genomic Target	Manufacturer
GI	Cog1F-flap	AATAAATCATAA CGYTGGATGCGNTTYCATGA	Norovirus GI	Integrated DNA Technologies
	Cog1R-flap	AATAAATCATAA CTTAGACGCCATCATCATTYAC		
GII	Cog2F-flap	AATAAATCATAA CARGARBCNATGTTYAGRTGGAT GAG	Norovirus GII	Integrated DNA Technologies
	Cog2R-flap	AATAAATCATAA TCGACGCCATCTTCATTCA		
NS5-2	NS5-2F	GAAGAGACCTGCGGCTCATG	WNV NS5-2	Integrated DNA Technologies
	NS5-2R	CGGTAGGGACCCAATTCACA		

Target	Probe	Sequence (5'- 3')	Genomic Target	Manufacturer
GI	Ring1a.2	6-FAM- AGATYGCGR/ZEN/ TCYCCTGTCCA -IBFQ	Norovirus GI	Integrated DNA Technologies
GII	Ring 2.2	JOE - TGGGAGGGY/ZEN/ GATCGCAATCT - IBFQ	Norovirus GII	Integrated DNA Technologies
NS5-2	NS5-2	TYE 665- CCA ACG CCA TTT GCT CCG CTG -IBRQ	WNV	Integrated

Probe	Probe		NS5-2	DNA Technologies
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Reagents:	Equipment:	Supplies:
PCR grade water	Biological Safety Cabinet Class 2	Pipette tips
Taqman® Fast Virus 1-Step Master Mix (Applied Biosystems, ThermoFisher cat# 4444432)	Pipettes	Microcentrifuge tubes
TE buffer	Applied Biosystems 7500 Fast Real-Time PCR System (with 7500 software v2.0.6)	MicroAmp® Fast Optical 96 well Reaction plates (0.1mL) (Applied Biosystems Cat# 4346906)
	Vortex Mixer	MicroAmp® Fast 8-tube strip (Applied Biosystems Cat# 4358293)
	Mini micro-centrifuge with 8-strip tubes adaptor	MicroAmp® Optical 8-Cap Strips (Applied Biosystems Cat# 4323032)
	Bench top PCR plate spinner	MicroAmp® 8-Cap Strip clear (Applied Biosystems Cat# N8010535)
	MicroAmp® Cap Installing Tool (Applied Biosystems Cat# 4330015)	MicroAmp® Optical Adhesive Film (Applied Biosystems Cat# 4311971)
		Isopropyl, 70% (Cat# 00023703)
		10% bleach (cat# 00050847)

IV. Quality Control:

A. Extraction Controls

1. Positive Extraction Control (PEC) – Norovirus GII positive control
2. Negative Extraction Control (NEC) – Norovirus negative controls
3. No Template Control (NTC) – molecular grade water

B. Amplification Controls

1. Extracted GI Positive Control – Extracted Norovirus GI positive RNA

V. Procedure:

A. Creating Norovirus Worksheet and Run Template for the 7500 Fast

	<div>Action</div> <div>Note: Perform manipulations of samples and controls in a genomic level PCR laboratory. Prepare primers and master mixes in a DNA –free room.</div>	Related Documents Title Number		
1	Create a sample plate map and master mix worksheet to be uploaded onto the ABI 7500 Fast.	NoroFast_Workflow_Template3		
	<i>Note:</i> See Noro and GI Worksheet Template for instructional details.			
2	Bring worksheet to ABI TaqMan computer Click on the 7500 Software icon, create a new experiment from the template Noro_and_GI.edt , and assign the plate name in the field where it says *Experiment Name on the Experiment Properties page.	PHMS-271-0001 Using the ABI TaqMan 7500		
3	Import the tab-delimited text file containing sample numbers for the run as follows: 1) Insert USB into the port of ABI 7500 computer workstation 2) Select “File”, “Import...”, “Browse” for the tab-delimited text file in the USB ex. (F:\Noro and GI\Run Import\YYYY) 3) Click on “Start Import” 4) Eject USB after file has been imported			
4	Ensure targets are defined as follows in the “Define Targets and Samples” tab of “Setup” under the Experiment Menu (shown):			
	Target Name	Reporter	Quencher	Color
	G1a.2	FAM	None	blue
	G2.2	JOE	None	green
	WNV_IPC	CY5	None	magenta
5	Under the “Assign Targets and Samples” tab (shown): 1) Ensure sample numbers have been imported correctly by cross referencing the Noro and GI worksheet 2) Ensure wells are correctly assigned detectors			
	If	Then		
	Targets must be edited	Select the affected well and check beside the appropriate targets under the “Assign target(s) to the selected wells” heading to the left.		

	Action				Related Documents Title Number
	Sample identifiers must be edited		Select the affected well(s) and edit sample identifiers as needed.		
6	Select the “Run Method” tab under “Run” in the Experiment Menu and ensure the run conditions are as shown:				
	Reverse Transcription	Initial Denaturation	40 Cycle Fast PCR		
	Hold	Hold	Step 1	Step 2	
	50.0 °C	95.0 °C	95.0 °C	60.0 °C	
	5:00	0:20	0:03	0:30	
	Proceed to procedure V.B. Preparing Master Mix for Norovirus Testing				

B. Preparing Master Mix for Norovirus Testing

	Action		Related Documents Title Number
1	Prepare clean and genomic rooms for use: turn on BSCs, ensure proper function, and decontaminate all working surfaces using 70% isopropanol.		PHMS-270-0001 Using Biological Safety Cabinets
2	Bring Noro and GI worksheet into clean room. Thaw sufficient amount of reagents, vortex briefly, and keep on a cold block during use. Record lot numbers of reagents used on the worksheet.		PHGI_203_20 03F11 Primers and Probes Mix QC Log Form NoroFast_Workflow_Template3
	If	Then	
	There is sufficient NoroFast 20x OligoMix	Proceed to the next step.	
	There is insufficient NoroFast 20x OligoMix	<ol style="list-style-type: none"> 1) Prepare 20x NoroFast OligoMix as per the recipe in Appendix A 2) Gently vortex and spin down. 3) Pipet 20µL aliquots into amber micro-centrifuge tubes 5) Label aliquots with “20x NoroFast OligoMix”, date, batch, initials and expiry date 6) Store at -20 °C, protected from light and freeze-thaws 	

3	Prepare enough NoroFast qRT-PCR mastermix for all samples and controls according to the amounts calculated in the Noro and GI Panel worksheet. (Table below illustrates the amounts for 20 reactions)			Using Variable Volume Air Displacement Pipettes, Ordering Oligonucleotide Probes Online, PHMS-412-0001, Ordering Oligonucleotide Primers Online, PHMS-411-0001, Reconstituting Oligonucleotide Probes, PHMS-412-0021, Reconstituting Oligonucleotide Primers, PHMS-412-0021, NoroFast Workflow Template	
	Reagent	x1 20uL rx (uL)	x 20 rx's mix (uL)		Final Conc.
	dH ₂ O	9	180		N/A
	4x FastVirus 1-Step Master Mix	5	100		1X
	20x NoroFast OligoMix	1	20		1X
	Total	15	300		N/A
	Note: Mastermix can be stored at 4 C in PCR clean fridge until ready to be used.				
4	Gently vortex master mix, spin down briefly, and aliquot 15 µL per well, for each sample to be tested, into a Fast optical 96 well plate (or Fast 8-tube strip). Note: Fast Optical plates must be used on Fast blocks as they provide the correct well volumes and fit (i.e. regular 96 well plates, whether Optical or not, are not interchangeable).				
5	Lightly cover the wells with a temporary clear plastic 96-well plate film (or clear 8-cap strip if using tubes) and transport the plate/tube into a genomic level PCR room. Note: Alternatively, you may use a Ziploc bag to transport your plate without using a plate seal or 8-well strips capped.				
	If		Then		
	Samples will be added immediately		Proceed with the next step.		
	Samples will not be added immediately		Store the plate/tube, protected from light, at 4°C until ready to inoculate template		
	Note: Avoid contaminating plate/tube with dust, oil or other fluorescent contaminants by handling edges with powder-free gloves and not placing plate onto any surface other than a BSC or clean surface.				
6	Thaw extracted GI positive control, briefly vortex and spin down.				

7	<p>Add template to the reactions as follows:</p> <p>1) Pipet 5 µL of extracted RNA to the corresponding well(s) for each extraction control and patient sample in the order on the Noro and GI Panel worksheet</p> <p>2) Add 5 µL PCR grade water to the corresponding NTC well</p> <p><i>Note: Visually check to be certain all reagent and sample levels are equal.</i></p>	PHGI_200_2001 Preparing Clinical Samples for Nucleic Acid Extraction	
8	<p>Carefully seal plate edges using a piece of MicroAmp® Optical Adhesive Film and avoid touching the top of the film. Conversely, seal the 8-tube strip with an optical 8-cap strip.</p> <p><i>Note: Ensure the wells/tubes are properly sealed against evaporation.</i></p>		
9	<p>Wearing clean gloves and handling the plate/tube by the edges, briefly vortex and spin down the plate/tube using a bench top PCR plate spinner/microcentrifuge.</p>		
	If	Then	
	Plate/tube will be run immediately	Proceed to the next step.	
	Plate/tube will be not run immediately	Store at 4°C, protect from light, until ready to run the plate.	
10	<p>Transfer the plate/tube to the ABI 7500 Fast.</p> <p>Note: Avoid contaminating plate/tube with dust, oil or other fluorescent contaminants by handling edges with powder-free gloves and not placing plate onto any surface other than a BSC or clean surface.</p>		
11	<p>Pull out the tray from the ABI 7500 Fast and insert the plate/tube in the correct orientation - handling by the edges only – and close the sample tray.</p>		Using the ABI 7500 PHMS-271-0001
12	<p>Select the “Start Run” button on the plate document to initiate the run and confirm that the run has started, “Run Status = Running”.</p> <p>Save the run in the appropriate folder on the 7500 Fast computer when prompted.</p> <p>Ex. (D:\AppliedBiosystems\SDSDocuments\Noro and GI\YYYY)</p> <p>When the “Run Status” reaches “In pre-cycling stage”, the “Estimated Time Remaining” will show and it should be around 58 minutes.</p>		Using the ABI 7500 PHMS-271-0001

C. Result Export and Analysis

1	Action	Related Documents Title Number	
2	<p>When the run is finished (i.e. “Estimated Time Remaining” indicator reads 00:00:00), Select the entire plate by clicking the upper left hand square of the 96 well plate and select “Analyze”.</p> <p>Note: “Analysis” will be selected under the Experiment Menu at this point, with the “Amplification Plot” tab selected by default.</p>	PHGI_203_2002 Using the ABI Taqman 7500 Fast Real-Time PCR System for the Detection of Norovirus	
3	Pull out the sample tray from the 7500 Fast, remove the used plate/tube, close the sample tray, and examine the wells and seal integrity.		
	If	Then	
	The reaction volume in one or more of the wells is significantly lower than the others	Notify a supervisor.	
	The plate seal or tube cap has lifted or appears to be otherwise compromised		
4	Ensure the following parameters are selected under “Amplification Plot” in the “Analysis” tab and select the “Analyze” button:		
	Tab	Parameter	Selection
	Plot Settings	Plot Type	ΔRn vs Cycle
		Graph Type	Log
		Plot Color	Target
	Options	Target	All
		Threshold	checked (✓)
Baseline Start		unchecked (□)	
5	Ensure that G1a.2 and G2.2 target thresholds are manually set to 0.1 and that the WNV_IPC threshold is manually set to 0.02.	PHGI_203_2002 Using the ABI Taqman 7500 Fast Real-Time PCR System for the Detection of Norovirus	

6	<p>View Control results as follows:</p> <ol style="list-style-type: none">1) Select the respective well in the 96-well plate to display the corresponding amplification curve in the Amplification Plot and note the Ct displayed in the well.2) View all of the targets in the amplification plot individually or simultaneously by selecting the target or “All” in the “Target” drop-down box.3) Verify the amplification results by selecting “Multicomponent Plot” <p>Note: The Legend under the Amplification Plot displays the color of each target's threshold and amplification curve line.</p>	
7	<p>Export the run results as a .xls file as follows:</p> <ol style="list-style-type: none">1) Insert USB into the port of ABI 7500 Fast computer workstation2) In the “View Plate Layout” tab, use the mouse and Ctrl key to highlight <u>only</u> the wells containing samples3) In the header toolbar, click on the “Export” (globe with arrow icon) and “Export...”4) In the “Export Data” dialogue box, ensure that:<ol style="list-style-type: none">a) “Export Properties” tab is selectedb) “Select data to export”: “Results” is checkedc) “Select one file or separate files:” “One File” is selectedd) “File Type”: *.xls is selected5) Beside “Export File Name:”, ensure that the proper assigned file name is entered6) Browse to the file location you want to save the file in beside “Export File Location” (F:\Noro and GI\Result Export\YYYY)7) Select “Start Export”8) Select “Close Export Tool” in the “Export Completed” dialogue box9) Safely remove USB <p>Note: Select only those wells containing sample for export of results. Avoid importing results from wells that do not contain sample – otherwise the ResultWizard will not display results correctly.</p>	
8	<p>Visualize the run results in the Noro and GI Panel Worksheet Template by pasting the exported results into the worksheet.</p> <p><i>Note: See Noro and GI worksheet for instructional details.</i></p>	
9	Confirm that the data exported correctly.	
	If	Then
	The file type is incorrect	Repeat the data export process
	The data range is incorrect	
The data exported correctly.	Click on the Report tab and print the collated results	

10	Determine if the controls worked as follows by entering into the “QC Stats Tool Norovirus” Ex. (H:\envbac\Molecular QA\QC Stats Tool Norovirus (Current).xslm)		PHQM_q07_0701 Using the QC Stats Tool to Collect Analyze Review Numeric QC Data
	If	Then	
	<ul style="list-style-type: none">The PEC Ct falls within the acceptable rangeThe NEC Ct is “undetermined”The NTC Ct is “undetermined”The PCR control Ct’s fall within the acceptable ranges	<ul style="list-style-type: none">The extraction process is in controlThe PCR process is in controlRecord the control Ct’s on the designated QC sheet or software.Proceed to sample analysis	
	The PEC Ct falls outside the acceptable range	<ul style="list-style-type: none">Notify a supervisorRepeat the PCR	
	A PCR control Ct falls outside its acceptable range		
	The NEC Ct is < 40 cycles		
The NTC Ct is < 40 cycles			
11	View each sample’s target Ct’s, confirming the amplification plots with the corresponding multicomponent plots.		
	If	Then	
	The Ct’s of all targets in the sample correspond to the amplification and multicomponent plot data	<ul style="list-style-type: none">The results are valid.Proceed to the next step.Interpret the sample’s target Ct’s as in Appendix B.	
	The Ct’s of all targets in the sample do not correspond to what is seen in the amplification and multicomponent plots	<ul style="list-style-type: none">The results may require operator interpretation.Consult a supervisor.Note the discrepancy in the “Comments” column for that sample on the printed results sheet.	
12	Report the patient sample results.		PHGI_304_3600_GIDO Result Entry in SmarTerm

VI. Procedure Notes:

N/A

VII. Method Limitations:

- A.** The levels of detection for the qRT-PCR targets are:
- GI: 10,000 copies per reaction
 - GII: 10,000 copies per reaction
 - WNV_IPC: 500 copies per reaction
- B.** Sample in which Norovirus GI or GII are present may have compromised Ct's at which WNV_IPC amplifies. As WNV_IPC serves as an indicator of inhibition and sample extraction, this is acceptable when target is detected in the sample.

VIII. References:

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- Zhuo, R., M.E. Hasing, Team of Molecular Diagnostics and X. Pang.** 2015. A single Nucleotide Polymorphism at the TaqMan® Probe-Binding Site Impedes the Real-Time RT-PCR detection of Norovirus GII.4 Sydney. *J. Clin. Microbiol.* **53**(10): 3353-3354.

IX. Appendix:

A: Recipe for NoroFast 20X OligoMix (pre-mix of all oligonucleotides required in NoroFast qRT-PCR). The recipe below is sufficient for 200 reactions.

20x OligoMix				Final Rx Conc (µM)
Stock Reagent (200µM)	x1 20uL reaction (µL)	Volume for 200 reactions (µL)	Conc in 20x OligoMix (µM).	
200 µM COG-1F	0.04	8	8	0.4
200 µM COG-1R	0.04	8	8	0.4
200 µM COG-2F	0.09	18	18	0.9
200 µM COG-2R	0.04	8	8	0.4
200 µM NS5-2F	0.01	2	2	0.1
200 µM NS5-2R	0.01	2	2	0.1
200 µM Ring1a.2 (FAM) Probe	0.02	4	4	0.2
200 µM Ring2.2 (JOE) Probe	0.02	4	4	0.2
200 µM NS5-2/WNV-IPC (TYE 665) Probe	0.02	4	4	0.2
TE	0.71	142	N/A	N/A
Total	1	200	N/A	N/A

B: Interpretation of Sample Target Ct's into Results

G1a.2 Ct (cycles)	G2.2 Ct (cycles)	WNV_IPC Ct (cycles)	Sample Result
Ct<35	Undetermined or Ct>40	N/A	Norovirus genogroup I Positive
Undetermined or Ct>40	Ct<35	N/A	Norovirus genogroup II Positive
Ct<35	Ct<35	N/A	Norovirus genogroup I and II Positive
Undetermined or Ct>40	Undetermined or Ct>40	> 5 Ct deviation from Neg Control Ct	Norovirus negative
35<Ct<40	Undetermined or Ct>40	> 5 Ct deviation from Neg Control Ct	RETEST SAMPLE RNA with undiluted and 1:10 dilution. If amplification curve is observed in repeat run then sample is Norovirus genogroup I POSITIVE.
Undetermined or Ct>40	35<Ct<40	> 5 Ct deviation from Neg Control Ct	RETEST SAMPLE RNA with undiluted and 1:10 dilution. If amplification curve is observed in repeat run then sample is Norovirus genogroup II POSITIVE.
35<Ct<40	35<Ct<40	> 5 Ct deviation from Neg Control Ct	RETEST SAMPLE RNA with undiluted and 1:10 dilution. If amplification curve is observed in repeat run then sample is Norovirus genogroup I and II POSITIVE.
Amplification observed at multicomponent curve but is below the threshold of 0.1 for G1a.2 or G2.2		N/A	Consult. Manual adjustment of target threshold to determine Ct for positivity.

Revision History

Version	Date	Writer	Description of Change
1.1	September 21, 2017	Stephanie Man	Unique identifier changed from PHGI_203_2003 to PHGI_255_2601 to align with new document identifier algorithm; updated worksheet template instructions. This SOP replaces PHGI_203_2003.