LightMix® Kit *Norovirus*

Cat.-No. 40-0361-96

Kit with reagents for the 1-step RT-PCR detection of *Norovirus GGI* and *GGII* RNA and for the detection of *Norovirus GGI* and *GGII* cDNA using the Roche Diagnostics LightCycler® 2.0 / 480 Instruments.

Lyophilized mix of primers and probes for a total of 96 reactions with a final volume of 20 µl each. **Store protected from light at room temperature (18-25°C), do N OT freeze!**

Instructions for use with the LightCycler® 2.0 Instrument see pages 4-5
Instructions for use with the LightCycler® 480 Instrument see pages 6-7

1. Set contents

1 Vial with blue cap containing premixed lyophilized primers and probes for 96 PCR reactions of *Norovirus GGI* and *GGII* and *MS2 IC*
1 Row with 6 lyophilized cloned plasmid DNA standards from 10^1 to 10^6 target equivalents per reaction of *Norovirus GGII*
1 Vial with colorless cap containing control DNA *Norovirus GGI,* 5 x 10^3 target equiv. per reaction
1 Vial with colorless cap containing control DNA *Norovirus GGII*, 5 x 10^3 target equiv. per reaction
1 Vial with purple cap containing IC DNA *MS2 phage*, 5 x 10^3 target equivalents per reaction
1 Sealing foil for the standard row

2. Additional reagents and items required

Roche Diagnostics:

LightCycler® FastStart DNA Master HybProbe	Cat.-No. 03 003 248 001
LightCycler® 480 RNA Master Hydrolysis Probes	Cat.-No. 04 991 885 001
RTR RNA Virus Master (LightCycler® 480 Instrument only)	Cat.-No. 05 619 416 001
LightCycler® Multicolor Demo Set (LightCycler® 2.0 Instrument only)	Cat.-No. 03 624 854 001
High Pure Viral Nucleic Acid Kit	Cat.-No. 11 858 874 001
RNA, MS2	Cat.-No. 10 165 948 001
LightCycler® Capillaries (20 µl) (LightCycler® 2.0 Instrument only)	Cat.-No. 04 929 292 001
LightCycler® 480 Multiwell Plate 384, white (LightCycler® 480 Instrument only with 384 well block)	Cat.-No. 04 729 749 001
or LightCycler® 480 Multiwell Plate 96, white (LightCycler® 480 Instrument only with 96 well block)	Cat.-No. 04 729 692 001

3. Product characteristics

PCR results are obtained within 40 minutes (45 cycles and melting curve) with the LightCycler® 2.0 Instruments and within 75 minutes (45 cycles and melting curve) with the LightCycler® 480 Instrument.

Sensitivity
These reagents detect 100 copies of *Norovirus* RNA using the Roche 'LightCycler® 480 RNA Master Hydrolysis Probes'.
With cDNA and the LightCycler® FastStart DNA Master HybProbe the detection limit is 10 copies (DNA).

Measuring range
The linear measuring range of the assay is 10^3 to 10^6 copies of *Norovirus GGI* DNA using the Roche 'LightCycler® 480 RNA Master Hydrolysis Probes'.

Storage and Stability
- Lyophilized reagents are stable for at least 3 months after shipment if stored protected from light at room temperature (18-25°C).
- **Do not freeze** lyophilized reagents.
- Dissolved reagents are stable for at least 5 days if stored protected from light and refrigerated (4°C).

4. Introduction

Norovirus, formerly Norwalk-like viruses, is a positive strand RNA virus with a high genomic variability. It can be classified into 5 different genogroups, from which most importantly *GGII* and to less extend *GGI* and GGIV infect humans. At least 50% of all gastroenteritis (stomach pain, diarrhea, and vomiting) are reported to be caused by *Norovirus*. RT-PCR detection of *Norovirus GGI* and *GGII* using the junction sequence between orf1 and orf2 has been published by Höhne and Schreier[1] and by Dreier et al.[2].

The LightMix® Kit *Norovirus* provides a fast, easy and accurate system to detect, quantify (needs external RNA standard) and genotype (differentiation between *GGI* and *GGII*, NOT possible with LightCycler® 1.x Instruments) this target in a nucleic acid extract.

A control amplification reaction based on MS2 RNA (or DNA) acts as internal positive control (IC).

This LightMix® Kit is tested on the LightCycler® 2.0 / 480 (96 well and 384 well formats) Instruments with Roche Diagnostics 'LightCycler® 480 RNA Master Hydrolysis Probes'.

[1]Detection of Norovirus genogroup I and II by multiplex real-time RT-PCR using a 3'-minor groove binder-DNA probe. Hoehne M and Schreier E. *BMC Infectious Disease* **6**:69 (2006).

[2]Enhanced Reverse Transcription-PCR Assay for Detection of Norovirus Genogroup I. Dreier J, Stormer M, Maede D, Burkhardt S, Kleesiek K. *J Clin Microbiol* **44(8)**:2714-2720 (2006).

5. Description

A 94 bp fragment of the junction between orf1 and orf2 is amplified with specific primers. The PCR product is detected with a fluorogenic DNA probe, resulting in an increase of the 530 nm signal.

The internal control (IC) is based on the PCR amplification of a fragment of the phage MS2 genome (read 610 nm signal). The IC can be run or on DNA target (as supplied in a separate vial with this kit) or on the genomic RNA (available from Roche Diagnostics, order number 10 165 948 001). The RNA based control allows to monitor the reverse transcriptase step and could be used as an extraction control (no protocol supplied).

The use of a color compensation file generated with the Roche Diagnostics 'LightCycler® Multicolor Demo Set' is a prerequisite to run the multiplex reaction (LightCycler® 2.0 Instrument only).

The supplied standard row of cloned and purified *Norovirus GGII* DNA allows to determine the linear range of the reaction and to estimate the quantity of the target sequence in unknown samples. The supplied control DNA of cloned and purified *Norovirus GGI* and *Norovirus GGII* DNA allows for the accurate comparison with unknown samples.

6. Experimental protocol

The following procedure was developed for use with the LightCycler® 2.0 / 480 Instruments.
Start programming before preparing the solutions. See the LightCycler® Instrument operator's manual for details.

Sample material: Use aqueous nucleic acid preparations (e.g. Roche Diagnostics 'High Pure Viral Nucleic Acid Kit').

Negative control: Always run at least one no-template control (NTC) - replace the template DNA with water.

Positive control: Run a positive control - replace the template DNA with the provided control DNA.
For quantification always use a fresh solution prepared from the provided standard row (single use only).

6.1. Preparation of parameter-specific reagents and reagents for the IC (96 reactions):

One reagent vial with a **blue** cap contains all primers and probes to run 96 LightCycler® reactions.

Add 100 µl PCR-grade water to each reagent vial, mix the solution (vortex) and spin down.

►**Use 1 µl** reagent for a 20 µl PCR reaction.

This solution is stable at least five days when stored refrigerated at 4°C. Avoid prolonged exposure to light.

6.2. Preparation of the standard row

The target DNA is provided in 6 different quantities to yield from 10^1 to10^6 target molecules in 5 µl once resolved. Start with the lowest concentrated standard (first tube from the extended lip). Use the pipette tip to punch a hole through the sealing foil. **Add 40 µl** PCR-grade water to each vial of the row. Mix the target DNA by pipetting the solution up and down 10 times.

►**Use 5 µl** standard for a 20 µl PCR reaction.

This standard solution is not long-term stable and will lose sensitivity under prolonged storage. Use only fresh prepared solutions as quantification references. Older solutions can be used as a qualitative standard (positive control). After adding the target DNA to the LightCycler® reaction mix use the provided sealing foil to close the vials in order to avoid changes in the concentration due to evaporation. Please note that opening these vials may cause contaminations of the work-space (aerosol).

6.3. Preparation of the control DNA

Add 100 µl PCR-grade water to each vial (10^5 target molecules) with a colorless cap. Mix the target DNA by pipetting the solution up and down 10 times (final conc.: 5×10^3 target molecules in 5 µl).

►**Use 5 µl** control DNA for a 20 µl PCR reaction.

This solution is stable at least five days when stored refrigerated at 4°C, for long term storage free ze at -20°C. Avoid repeated freezing thawing cycles. Please note that opening these vials may cause contaminations of the work-space (aerosol).

6.4. Preparation of the IC DNA

Add 100 µl PCR-grade water to the vial (5×10^5 IC-DNA molecules) with a purple cap. Mix the target DNA by pipetting the solution up and down 10 times (final concentration: 5×10^3 target molecules in 1 µl).

►**Use 1 µl** IC DNA for a 20 µl PCR reaction.

This solution is stable at least five days when stored refrigerated at 4°C, for long term storage free ze at -20°C. Avoid repeated freezing thawing cycles. Please note that opening these vials may cause contaminations of the work-space (aerosol).

6.5. Preparation of the LightCycler® reaction mix

In a cooled reaction tube, prepare the reaction mix by multiplying each volume for a single reaction by the number of reactions to be cycled plus one additional reaction.

For use with the 480 RNA Master Hydrolysis Probes	
Single reaction	Component
4.3 µl	water, PCR-grade (colorless cap, provided with the Roche kit)
1.3 µl	Activator, (50 mM $Mn(OAc)_2$,green cap, provided with the Roche kit
1.0 µl	**reagent** mix (parameter specific reagents containing primers and probes, see **6.1.**)
1.0 µl	**IC** DNA (or MS2 RNA)
7.4 µl	LightCycler® 480 RNA Master Hydrolysis Probes
15.0 µl	**Volume of reaction mix**

To include the internal positive control add 1 µl of the IC DNA per reaction to the reaction mix.
To run the assay without the internal control add additional 1 µl PCR-grade water instead of the IC DNA to the reaction mix.

Mix gently, spin down and **transfer 15 µl** each of the reaction mix to a LightCycler® capillary (LightCycler® 2.0 Instrument) or to a multiwell plate (LightCycler® 480 Instrument).

Add 5 µl of sample or control DNA/standard to each capillary or well for a final reaction volume of 20 µl.
Start run.

7. LightCycler® 2.0 Instrument

7.1. Programming

The protocol consists of five program steps

- 1: Reverse Transcription (RT): transcription of the RNA to cDNA
- 2: Denaturation: sample denaturation and enzyme activation
- 3: Cycling: PCR-amplification of the target DNA
- 4: Melting: melting curve analysis for identification of the PCR product derived from the target DNA
- 5: Cooling: cooling the instrument

Program Step:	RT	Denaturation	Cycling			Melting			Cooling
Parameter									
Analysis Mode	None	None	Quantification mode			Melting Curves mode			None
Cycles	1	1	45			1			1
Target [℃]	61	95	95	56	72	95	40	85	40
Hold [hh:mm:ss]	00:03:00	00:00:30	00:00:10	00:00:10	00:00:05	00:00:20	00:00:20	00:00:00	00:00:30
Ramp Rate [℃/s]	20	20	20	20	20	20	20	0.2	20
Acquisition Mode	None	None	None	Single	None	None	None	Continuous	None

For use with Roche 'LightCycler® FastStart DNA Master HybProbe' an initial denaturation of 10 minutes is necessary (2-step protocol with previous cDNA synthesis).

7.2. Data Analysis

Switch the color compensation mode on (LightCycler® 2.0 Instrument only). If this mode is not enabled run the color compensation program. Follow the instructions in the manual of Roche Diagnostics 'LightCycler® Multicolor Compensation Set'.

7.2.1. Virus Detection

Perform data analysis, as described in the LightCycler® Instrument operator's manual. We recommend using the Second Derivative Maximum method. The cycle number of the Crossing Point (CP) of each sample is calculated automatically. The Fit Points method is more-error prone due to the user's influence.

View *Norovirus* data in channel 530 Quantification mode.
The negative control (NTC) must show no signal.

View IC data in channel 610, Quantification mode. The negative control and the *Norovirus* samples should show an amplification curve for the IC with a CP at approximately cycle 35.

The provided standard row of cloned and purified DNA with concentrations in the range from 10^6 copies/rxn to 10^1 copies/rxn of *Norovirus GGII* should have CPs between cycles 20 and 27 (for 10^6 to 10^3 copies/rxn, CPs calculated with Second Derivative Maximum method).

7.2.2. Virus Genotyping

Run a melting curve experiment as described (7.1. Programming) using 'Tm calling' Analysis mode, channel 670. Samples with a melting signal are *Norovirus GGII*.

7.3. Sample Data – typical results

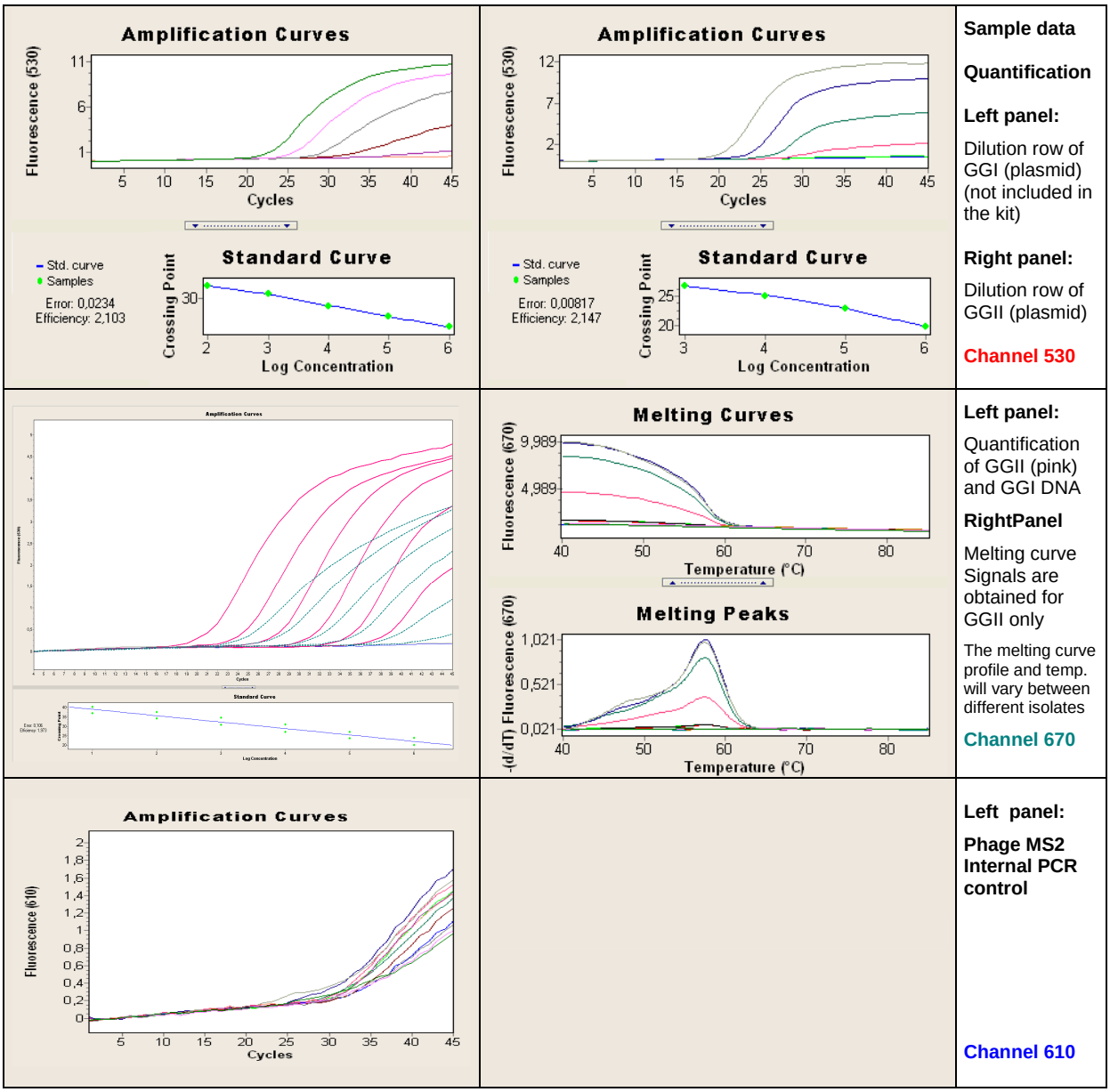

Fig.1. Sample data for the *Norovirus* detection system. Data from LightCycler® 2.0 Instrument

Upper panels: Left panel channel 530 quantification mode (Second Derivative Maximum) with calibration curve for *Norovirus GGI*. Right panel channel 530 quantification mode (Second Derivative Maximum) with calibration curve for *Norovirus GGII*.
Middle panels: Left panel GGII (pink) and GGI (Green) DNA amplification with the FastStart kit; detection limit is 10 copies. Right panel channel 670 melting analysis (Tm Calling) for *Norovirus GGII* The melting temperature is about 58℃ but can vary depending on the run conditions and virus isolate. Samples containing only *Norovirus GGI* will not generate a melting curve.
Lower panel: Internal control (IC) in channel 610 quantification mode (Second Derivative Maximum) on MS2 DNA target.

7.4. Interpretation of data

Norovirus channel 530	Internal Control channel 610	Melting Curve channel 670	NTC sample channel 530	Result
amplification	not relevant	**no signal**	**negative**	**Positive for *Norovirus GG I***
amplification	not relevant	**peak 50-60℃**	**negative**	**Positive for *Norovirus GG II***
no amplification	**amplification**	**no signal**	**negative**	**Norovirus not detectable**
no amplification	**no amplification**	**no signal**	not relevant	**PCR inhibition**, repeat experiment
amplification	amplification	not relevant	**positive**	**Contamination**, repeat experiment

Tab. 3. Typical analysis results (LightCycler® 2.0 Instrument, Roche Master: 480 RNA)

8. LightCycler® 480 Instrument

8.1. Programming

The protocol consists of four program steps

- 1: Reverse Transcription (RT): transcription of the RNA to cDNA
- 2: Denaturation: sample denaturation and enzyme activation
- 3: Cycling: PCR-amplification of the target DNA
- 4: Cooling: cooling the instrument

Detection Format:
LightCycler® 480 Instrument: 483-533, 558-610
LightCycler® 480 II Instrument: 465-510, 533-610

Program Step:	RT	Denaturation	Cycling			Cooling
Parameter						
Analysis Mode	None	None	Quantification mode			None
Cycles	1	1	45			1
Target [℃]	61	95	95	56	72	40
Hold [hh:mm:ss]	00:03:00	00:00:30	00:00:10	00:00:10	00:00:05	00:00:30
Ramp Rate [℃/s] **96**	4.4	4.4	4.4	2.2	4.4	1.5
Ramp Rate [℃/s] **384**	4.6	4.6	4.6	2.4	4.6	2.0
Acquisition Mode	None	None	None	Single	None	None
Acquisitions [per ℃]	-	-	-	-	-	-

8.2. Data Analysis

Note: For use on LightCycler® 480 II Instruments use Filter Combination 465-510 instead of Filter Combination 483-533, Filter Combination 533-610 instead of Filter Combination 558-610 and Filter Combination 498-660 instead of Filter Combination 483-670 for detection.

LightCycler® 480 I Instrument		LightCycler® 480 II Instrument
Excitation 483 – Emission 533 (FAM)		Excitation 465 – Emission 510 (FAM)
Excitation 558 – Emission 610 (ROX)		Excitation 533 – Emission 610 (ROX)
Excitation 483 – Emission 670 (LC670)		Excitation 498 – Emission 660 (LC670)

8.2.1. Virus Detection

Perform data analysis, as described in the LightCycler® Instrument operator's manual. We recommend using the Second Derivative Maximum method. The cycle number of the Crossing Point (cp) of each sample is calculated automatically. The Fit Points method is more-error prone due to user's influence.

View *Norvirus* data with Filter Combination 483-533, Quantification mode.
The negative control (NTC) must show no signal.

View IC data with Filter Combination 558-610, Quantification mode. The samples should show an amplification curve for the IC with a CP at approximately cycle 35.

The provided standard row of cloned and purified DNA with concentrations in the range from 10^6 copies/rxn to 10^1 copies/rxn of *Norovirus GGII* should have CPs between cycles 20 and 27 (for 10^6 to 10^3 copies/rxn, CPs calculated with Second Derivative Maximum method).

8.2.2. Virus Genotyping

Run a melting curve as separate experiment (if using the LightCycler® 480 Instrument) with the Detection Format 'Multi Color HybProbe'. Program 30 sec 95℃ denaturation, 120 sec 40℃ hybridization, melt from 40℃ to 85℃ with 3 a cquisitions per ℃, cooling for 10 sec at 40℃. View data with Filter Combination 483-670 (version II: Filter 498-660), 'Tm calling' Analysis mode. Samples showing a melting signal are from *Norovirus genogroup II*. In case that the melting curve analysis shall be included in the LightCycler® 480 run please ask for detailed instructions for a specific color compensation.

Melting			Cooling
Color Compensation			None
1			1
1	2	3	1
95	40	85	40
00:00:30	00:02:00	00:00:00	00:00:30
4.4	*1.5*	-	*1.5*
4.6	*2.0*	-	*2.0*
None	None	Cont	None
-	-	1	None

8.3. Sample Data – typical results

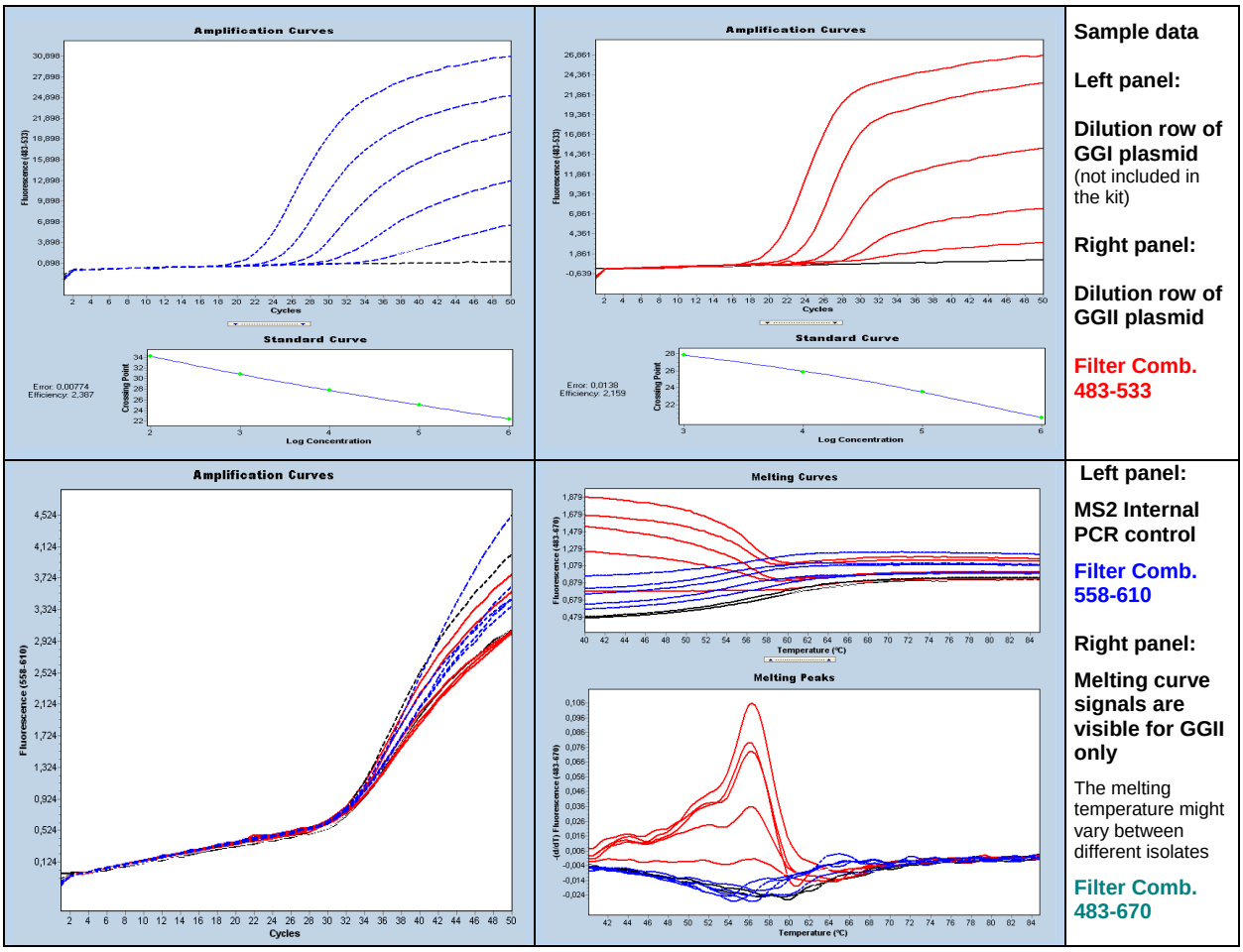

Fig.1. Sample data for the *Norovirus* detection system.

Upper panels: Data from LightCycler® 480 Instrument Filter Combination 483-533. Left panel data for *Norovirus GGI* quantification mode (Second Derivative Maximum). Right panel data for *Norovirus GGII* quantification mode (Second Derivative Maximum). The standard row shows amplification signals from 10^2 to 10^6, NTC and 10^1 show no amplification signals.
Lower panels: Data from LightCycler® 480 Instrument. Left panel data for the MS2 internal positive control, Filter Combination 558-610 quantification mode (Second Derivative Maximum).
Right panel data for *Norovirus GGII,* Filter Combination 483-670 melting analysis (Tm Calling).

9. Interpretation of data

Norovirus filter comb. 483-533	Internal Control filter comb. 558-610	Melting Curve filter comb. 483-670	NTC sample filter comb. 483-533	Result
amplification	not relevant	**no signal**	**negative**	**Positive for *Norovirus GG I***
amplification	not relevant	**peak 50-60℃**	**negative**	**Positive for *Norovirus GG II***
no amplification	**amplification**	**no signal**	**negative**	**Norovirus not detectable**
no amplification	**no amplification**	**no signal**	not relevant	**PCR inhibition**, repeat experiment
amplification	amplification	not relevant	**positive**	**Contamination**, repeat experiment

9. Notes / Error Prevention Measures / Updates

The supplied internal control target (MS2 phage genomic fragment, cloned DNA) has been removed from the pre-mixed reagents and supplied in a separate vial to enable to use MS2 genomic RNA as IC target (06-2008).

Previous lots of this product showed sometimes false positive results in channel 530 (FAM). Since a melting curve signal in channel 670 was missing these samples were interpreted as GG1 virus.

The signals in channel 530 were caused by unspecific reactions between the GG1 forward primer and the GG2 detection FAM probe. The sequence of the GG1 primer has been modified slightly (10-2008).

Increasing the PCR annealing temperature from 52℃ to 55℃ - 58℃ avoids the unspecific interaction and solves the problem (11-2008).

The primers for the detection of the internal control (IC) had to be changed to generate an adequate signal also at higher temperatures (upgrade 11-2008).

For viewing GG2 amplification in the 670 channel run the Norovirus kit at 55℃ annealing temperature. This is only an option; the recommended procedure is reading channel 530 for Norovirus detection and performing a melting curve analysis to identify GG2 virus.

These reagents were developed and manufactured by TIB MOLBIOL GmbH, Berlin, Germany.
SimpleProbe® and LightCycler® hybridization probes made under license from Roche Diagnostics GmbH (worldwide ex USA).