# LightMix® Modular Gastro Virus Multiplex Testing

## 1. Intended Use

The products listed in section 3 are intended to be used for the identification of diarrhea causing pathogens (qualitative assays). Parasitical genomic DNA is detected in nucleic acid (NA) extracted from watery stool samples obtained from patients with diarrhea. These products can be utilized for multiplex or single pathogen genome detection. Molecular test results should not be used as the only basis for patient treatment decisions.

## 2. Introduction

Infectious diarrhea can be caused by viruses, bacteria or parasites. These instructions describe the use of LightMix® Modular Kits for single or multiplex PCR genome detection of pathogens, using Roche 480 amplification instruments (as listed in section 6).

**LightMix® Modular (ModularDx) Kit General Information**. Up to six channels can be utilised simultaneously to maximise throughput, enabling the detection of five targets and a control in a single reaction. The 660 (Cy5) channel is reserved for the Control Reaction. The panel can be customized to the individual laboratory testing requirements; single assays can be run or panels can be built by combining, omitting or replacing individual assays. In addition to target selection the laboratory can choose to include an internal control, a spiked extraction control, or a Roche Process Control (RPC/DPC), to monitor the entire process from extraction, reverse transcription (where applicable) to amplification, detection and result interpretation.

## 3. LightMix® Modular Kits Components and Ordering Information

| | | | | channel |
|---|---|---|---|---|
| 50-0652-96 | 07792239001 | Norovirus GG1 | CE-IVD | 500 |
| 53-0652-96 | 07792247001 | Norovirus GG1 | CE-IVD | 530 |
| 53-0653-96 | 07792255001 | Norovirus GG2 | CE-IVD | |
| 58-0652-96 | 07915110001 | Norovirus GG1 | CE-IVD | 580 |
| 58-0654-96 | 07889887001 | Rotavirus A | CE-IVD | |
| 61-0909-96 | 07654235001 | EAV | Extraction Control (RNA) | 610 |
| 66-0909-96 | 07374330001 | EAV | Extraction Control (RNA) | 660 |
| 90-0600-01 | 07932146001 | Gastro Pos Control | | |

## 4. Multiplex PCR Combinations (examples)

**Gastro Virus Multiplex PCR**

Color Compensation 40-0320 is mandatory for Multiplex PCR

| 500 | 530 | 580 | 610 | 640 | 660 | 480 II | z 480 | |
|---|---|---|---|---|---|---|---|---|
| | GG1+GG2 | | | | Control | X | X | Duplex |
| | GG1+GG2 | | Control: EAV | | | X | X | Duplex |
| GG1 | GG2 | | | | | X | | Triplex |
| | GG2 | GG1 | | | | X | X | Triplex |
| GG1 | GG2 | RotaV A | pathogen 4 | | EAV or RPC | X | | 5plex |
| GG1 | GG2 | RotaV A | pathogen 4 | pathogen 5 | | X | | 6plex |

The **cobas z** 480 analyzer cannot discriminate 500 and 530 as distinct channels; select either one kit or read the information for both kits in the FAM channel (procedure not described in this manual).

## 5. LightMix® Modular Kit Storage

Kits are shipped without cooling. Tropical Climate Transport Simulation Stability studies indicate that these products remain stable after three days storage at 60°C.

**Upon arrival** store kits cooled or at room temperature (4°C to 25°C). **Do not freeze lyophilized reagents.** Store in the dark. Lyophilized kits are stable for one year after production. See lot-specific expiry date.

**Open Vial Stability / On-board Stability**

**Once dissolved** positive controls must be stored frozen (-15°C to -25°C). **Record date of first use**.

**Once dissolved** single or mixed target reagents can be stored **for daily use** for up to 30 days refrigerated. For longer term storage freeze at -15°C to -25°C until expiry. Minimize repeat freeze-thaw cycles (< 10). **Record** use.

To minimise the potential for errors associated with self-labeled tubes, do not transfer to secondary vials.

## 6. Additional Reagents Required and Instrument Information

| | | |
|---|---|---|
| LightCycler® Multiplex RNA Virus Master | | Cat.-No. 06 754 155 001 |
| RNA Process Control Kit | (optional) | Cat.-No. 07 099 592 001 |
| LightCycler® 480 II Instrument | | Cat.-No. 05 015 278 001 |
| cobas z 480 Analyzer, UDF software 1.5 | | Cat.-No. 05 200 881 001 |
| LightCycler® 480 Multiwell Plate 96 white or | | Cat.-No. 04 729 692 001 |
| LightCycler® 480 Multiwell Plate 384 white | | Cat.-No. 04 729 749 001 |
| Color Compensation Kit Hexaplex 40-0320-00 | | Cat.-No. 06 296 971 001 |
| Bovine Serum Albumin (BSA) 20 µg/µl | | Cat.-No. 10 711 454 001 |

## 7. Precautions and Warnings

**Handling Requirements**
The product is an *in-vitro* diagnostic device and therefore must be used by qualified personnel only.
Before using this product, read the operator / safety instructions in the instruments operator's manual.

General precautions for the handling of samples and generic laboratory materials are required.

The laboratory work-flow must conform to standard practices. Due to the risk of contamination, PCR preparation and PCR amplification must be performed in physically separated areas.

Do not mix reagents from different lots. Do not combine assays other than described in this manual.

Do not use reagents after the expiration date. Use the manual version valid for the kit in use (see kit label).

**Laboratory Procedures**
All materials of human origin and related waste must be considered potentially infectious. Thoroughly clean and treat all work surfaces with disinfectants approved by local authorities.

Do not eat, drink or smoke in the laboratory. Do not pipette by mouth.

Wear disposable protective gloves, laboratory coats and adequate eye protection during the handling of samples and reagents. Thoroughly wash your hands after handling the samples and reagents.

Avoid microbial or nuclease contamination of the reagents. The use of disposable filter tips is mandatory.

**Handling of Waste Materials**
Dispose of the unused reagents and inactivate waste materials according to the current local guidelines.

**Result Interpretation and Reporting**
The use of a color compensation file generated with the LightMix® Hexaplex Color Compensation kit is a prerequisite to perform multiplex PCR reaction result interpretation. Result interpretation involves a general-use software which has not specifically been adapted for analysis of these assays. Software 'positive' and 'negative' result calls must be verified by the operator to avoid the reporting of false results.

Molecular testing reports genome-based results indicating the presence of a pathogen. Molecular test results should not be the only basis for a treatment decision. PCR methodologies are extremely sensitive and may generate more positive results than traditional methodologies; the Cp values can help to indicate the significance of the reported pathogen, in particular where multiple infections are detected. Contamination events can cause false positive results.

# 8. Background Information

## 8.1. Medical Background

Diarrheal bowel movements are loose and watery. Diarrhea is common, with many people experiencing diarrheal bowel movements a few times per year; for those with irritable bowel syndrome it may occur more frequently. Diarrhea is generally not a serious condition, however elderly or young children may suffer from dehydration. Serious symptoms are blood, mucus, or undigested food in the stool, loss of weight and fever.

Diarrhea is defined as three or more loose stools per day and has a number of causes: infection, inflammation and malabsorption. Infectious diarrhea is a leading cause of morbidity and mortality worldwide, the burden is especially great in young children in developing countries. Infectious diarrhea is commonly caused by viruses and less often bacteria (food poisoning) or parasites. It is transmitted by ingestion of contaminated food or water or person-to-person contact. Gastroenteritis caused by parasites is often more chronic in nature lasting >10 days, with symptoms that may include fever, abdominal pain, and bloating. Diarrhea may be bloody (dysentery), watery, or greasy. Some pathogens cause invasive disease.

There are currently no antiviral therapies available, however some bacterial and parasitical infections can be treated. Diagnosing the cause for diarrhea can assist clinicians in choosing the appropriate therapy.

Diarrhea can also be caused by malabsorption of certain nutrients, lactose intolerance, allergies to certain foods, diabetes, medication, radiation therapy, drug and alcohol abuse, or diseases of the intestinal tract (e.g. ulcerative colitis, Crohn's disease).

## 8.2. Methodology and Assay Principle

During the extraction process all cells and viruses contained in the sample are lysed. The genomic Nucleic Acids (RNA and/or DNA) are extracted and purified for detection utilizing PCR based amplification. LightCycler® amplification Reagent Kits are designed to incorporate dUTP into the PCR products, enabling the use of Uracil-DNA Glycosylase to prevent carry-over contamination (DNA assays only).

Each kit contains primers designed to specifically amplify one or more short fragments of the respective pathogen genome. The PCR fragments are detected using dual labeled hydrolysis probes, which generate a light signal during sequence-specific cleavage that is detected by the amplification instrument. The cycle number (Cp) where the signal exceeds the detection threshold, is proportional to the negative logarithm of the amount of starting material, enabling an estimation of the amount of pathogen in the sample.

The hydrolysis probes contain one of six possible dye labels (see section 3), enabling the detection of up to six different target reactions simultaneously. A Control Reaction in the 660 channel should be included in each multiplex reaction, to monitor lysis (Roche Process Controls only), extraction, and amplification.

## 8.3. Sample Collection and Nucleic Acid Extraction

Collect samples in clean containers without additives; samples must not be contaminated with urine or toilet paper. Stool samples should be transported and stored at 4°C to 10°C but not frozen. Prepare 10% (w/v) fecal suspensions by adding (dependent on the consistency) approximately 50-100 mg or 100 µl stool sample to 1 ml of Phosphate Buffered Saline (PBS) or S.T.A.R. buffer. Store residual specimens cooled at 4°C to 10°C for confirmation by subsequent culture or microscopy if the sample had a positive PCR result.

Homogenize fecal suspensions by vortexing for 1 minute, incubate at room temperature (optional 95°C) for 10 mins, and repeat vortexing. Centrifuge for 30s at 1,000 x g and transfer the supernatant to a new tube. Add the extraction control target (PhHV, EAV, or Roche RPC) to a final sample volume of 200 µl into a MagNA Pure processing cartridge ***or*** (alternative procedure) add the control target to the lysis buffer, then start the extraction.

**Optional Sample Pre-processing**. Physical disruption of cells with beads or freeze-thaw cycles (liquid nitrogen (-80°C) and heating to 95°C) has been published to improve the extraction yield, particularly for detection of parasites or worms (eggs).

The ModularDx Kits have been evaluated for use with nucleic acid extracted on the Roche Diagnostics MagNA Pure 96 instrument, using the 'DNA and Viral NA Small Volume Kit', Pathogen Universal or Viral NA Plasma SV protocol, 200 µl input and 50 µl/100 µl elution volume, following the MagNA Pure instrument instructions. Extraction with the MagNA Pure 24 instrument is expected to yield equivalent results.

**Roche recommendation**: For extracts derived from stool samples amplified with the Roche LC Multiplex Master mixes add 0.2 µg/µl (final) of Bovine Serum Albumin (BSA) to the PCR reaction (section 13.3.3).

# 9. Pathogen/Target Information - Analytical Specificity/Sensitivity

## 9.1. Norovirus

***Norovirus*** is the most common cause of viral gastroenteritis in humans, transmitted by contaminated food or person-to-person contact. Outbreaks occur often in closed communities such as schools or on cruise ships. Symptoms arise after one to two days and last for a couple of days; the disease is usually self-limiting, Norovirus infection is characterized by nausea, vomiting, watery diarrhea, abdominal pain; lethargy, weakness, muscle aches, headaches, and low-grade fevers may occur.

*Norovirus* is a positive strand RNA virus with a high genomic variability. classified into 5 genogroups (GG), from which GG2 and to less extend GG1 (or GG4) infect humans.

A non-functional fucosyltransferase FUT2 provides high protection from the most common norovirus GII.4.

GG1
A 70 bp long fragment from the *NoV* junction sequence between the orf1 and orf2 genes is amplified with specific primers and detected with a Cyan500 (500 channel) or FAM (530) labeled hydrolysis probe.

GG2
A 95 bp long fragment from the *NoV* junction sequence between the orf1 and orf2 genes is amplified with specific primers and detected with a FAM labeled hydrolysis probe (530 channel).

The LOD determined with *in-vitro* and viral RNA for different subytpes was 12 copies/rxn or better.

## 9.2. Rotavirus

**Rotavirus** infections are the most frequent cause of severe diarrhoea in children; adults are normally immunized from previous infections. The virus is faecal-oral transmitted. Infections are more common in the winter time. The virus has a double stranded RNA genome which is difficult to denature prior PCR amplification. From the five species of Rotavirus A-E most infections are caused by type A.

A 87 bp fragment from the NSP5 gene (segment 11) is amplified with specific primers and detected with specific R6G labeled hydrolysis probes (580 channel).

The LOD determined with *in-vitro* and viral RNA was 4.5 (CI 3.8-5.6) copies/rxn.

## 9.3. Summary Analytical Specificity / Cross-Reactivity

**Analytical Specificity**: Other bacteria, parasites or viruses which may be present in NA extracts from stool have been tested negative :

| Pathogen | Sapovirus | Norovirus | Rotavirus | Adenovirus | Astrovirus | Enterovirus |
|---|---|---|---|---|---|---|
| Sapovirus | **positive** | negative | negative | negative | negative | negative |
| Norovirus GG1 | negative | **positive** | negative | negative | negative | negative |
| Norovirus GG2 | negative | **positive** | negative | negative | negative | negative |
| Rotavirus | negative | negative | **positive** | negative | negative | negative |
| Adenovirus | negative | negative | negative | **positive** | negative | negative |
| Astrovirus | negative | negative | negative | negative | **positive** | negative |
| Enterovirus | negative | negative | negative | negative | negative | **positive** |
| *Yersina* | negative | negative | negative | negative | negative | negative |
| *Campylobacter* | negative | negative | negative | negative | negative | negative |
| *Shigella* | negative | negative | negative | negative | negative | negative |
| *Salmonella* | negative | negative | negative | negative | negative | negative |
| *Aeromonas* | negative | negative | negative | negative | negative | negative |
| *Plesiomonas* | negative | negative | negative | negative | negative | negative |
| *Bacillus cereus* | negative | negative | negative | negative | negative | negative |
| *Bacteroides* | negative | negative | negative | negative | negative | negative |
| *Helicobacter* | negative | negative | negative | negative | negative | negative |
| *Clostridium spp* | negative | negative | negative | negative | negative | negative |
| *Vibrio chol.* | negative | negative | negative | negative | negative | negative |
| *Entamoeba* | negative | negative | negative | negative | negative | negative |
| *Giardia* | negative | negative | negative | negative | negative | negative |
| *Dientamoeba* | negative | negative | negative | negative | negative | negative |
| *Cryptosporidium* | negative | negative | negative | negative | negative | negative |
| *Blastocystis* | negative | negative | negative | negative | negative | negative |
| *Schistosoma* | negative | negative | negative | negative | negative | negative |
| *Ancylostoma* | negative | negative | negative | negative | negative | negative |
| *Strongyloides* | negative | negative | negative | negative | negative | negative |

# 10. Test Specification - Certificate of Analysis

The Analytical Sensitivity has been determined for both single and/or multiplex PCR. Each lot is verified to detect at least 10 target copies per 20 µl reaction in a single PCR reaction (product specification).

Lot-specific signal levels (normalized to a manufacturer internal reference) and lot-specific Cp values for each target amount (plasmid positive control) with cut-off values derived from the value expected for 2-5 copies are printed in the lot-specific Certificate-of-Analysis (CoA) included with each product.

# 11. Evaluation Studies

## 11.1 Norovirus

A German study compared 183 sample against kit RB, running a multiplex PCR for NoV GG1 / GG2 combined with EV, RotaV A, AdV F, and AstV, reporting 46 pos/pos, 9 false pos, 1 false neg, and 127 neg/neg results, corresponding to 97.9% and 93.4% Sensitivity and Specificity.

An Australian study compared 173 samples against kit SG, running a multiplex PCR with RotaV and AdV F with 53 pos/pos, 1 false neg and 119 neg/neg results, Sensitvity/Specificity 98.1%, and 100%.

## 11.2 Rotavirus

A German study compared 183 sample against kit RB, running a multiplex PCR for NoV GG1 / GG2 combined with EV, RotaV A, AdV F, and AstV, reporting 46 pos/pos, 11 false pos, no false neg, and 126 neg/neg results, corresponding to 100% Sensitivity and 97.1% Specificity.

# 12. Evaluation Data Summary

Summary of evaluation results :

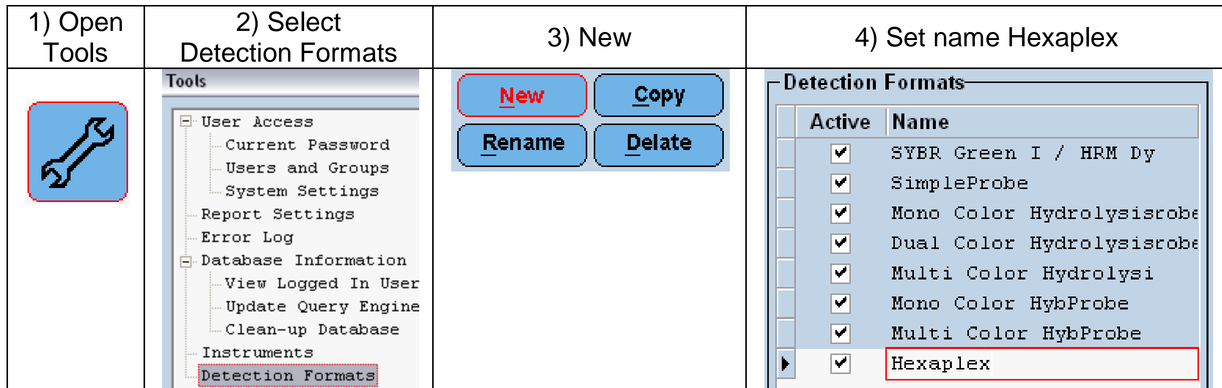
| **Channel**<br>**Pathogen** | **500**<br>**GG1** | **530**<br>**GG1** | **530**<br>**GG2** | **530**<br>**GG1+GG2** | **580**<br>**Rotavirus** |
|---|---|---|---|---|---|
| $LOD^{single}$ (copies/rx) | < 10 | <10 | <10 | <10 | <10 |
| $LOD^{multiplex}$ (copies/rx) | 113 | 113 | 4.5 | 12 | 4.5 |
| 95% CI | 100-127 | 100-127 | 3.8-5.6 | nd | 3.8-5.6 |
| Diag. Sensitivity | nd | nd | nd | 97.7% | 100% |
| Diag. Specificity | nd | nd | nd | 93.4% | 97.1% |
| Prevalence | nd | nd | nd | 10.4% | 10.7% |
| PPV | nd | nd | nd | 83.6% | 80.7% |
| NPV | nd | nd | nd | 99.7% | 100% |
| Intra Assay Variation | 0.63 | 0.63 | 0.84 | nd | 0.84 |
| Inter Assay Variation | 1.37 | 1.37 | 2.33 | nd | 2.33 |
| Inter Lot Variation | 0.85 | 0.85 | 1.17 | nd | 1.17 |

n.d. = not determined

# 13. Instructions for Use

See the Instrument operator's manual for details. Start programming before preparing the solutions.

## 13.1. Dye Channels and Color Compensation




Define Emission and Excitation filters for channels 500, 530, 580, 610, 640 and 660 (channel 500 is not available for cobas z 480 analyzer). Ensure a valid color compensation (cc) file is available for application; see the manual: ***LightMix® 40-0320 Universal Color Compensation Hexaplex*** for detailed instructions on performance of cc experiments. Set all parameters as described below:

| Name | 500 | 530 | 580 | 610 | 640 | 660 |
|---|---|---|---|---|---|---|
| LightCycler® 480 | **450-500** | **483-533** | **523-568** | **523-610** | **523-640** | **615-670** |
| LightCycler® 480 II | **440-488** | **465-510** | **533-580** | **533-610** | **533-640** | **618-660** |
| cobas z 480 | **n.a.** | **465-510** | **540-580** | **540-610** | **540-645** | **610-670** |
| Quant Factor * | **10** | **10** | **10** | **10** | **10** | **10** |
| Max Integration Time* | **1 sec** | **1 sec** | **1 sec** | **2 sec** | **3 sec** | **3 sec** |

* Adaption of instrument settings is allowed (under the responsibility of the operator).

## 13.2. Programming Roche '480' Instruments

For use with Roche '480' instruments, software 1.5 and higher. See the Instrument operator's manual for details. Program the instrumentation prior to reagent preparation.

The protocol consists of four program steps:

- 1: Reverse Transcription of the viral RNA
- 2: Denaturation: sample denaturation and enzyme activation
- 3: Cycling: PCR-amplification
- 4: Cooling: cooling the instrument

| **Program Step:** | ***RT Step*** | ***Denaturation*** | ***Cycling*** | | | ***Cooling*** |
|---|---|---|---|---|---|---|
| **Parameter** | | | | | | |
| Analysis Mode | ***None*** | None | Quantification mode | | | None |
| Cycles | *1* | 1 | 45 | | | 1 |
| Target [°C] | 55 | 95 | 95 | 60 | 72 | 40 |
| Hold [hh:mm:ss] | 00:05:00 | 00:05:00 | 00:00:05 | 00:00:15 | 00:00:15 | 00:00:30 |
| Ramp Rate [°C/s] **96** | 4.4 | 4.4 | 4.4 | 2.2 | 4.4 | 1.5 |
| Ramp Rate [°C/s] **384** | 4.6 | 4.6 | 4.6 | 2.4 | 4.6 | 2.0 |
| Acquisition Mode | None | None | None | **Single** | None | None |

 © 2017 TIB MOLBIOL 

## 13.3. Experimental Protocol

- **Sample material:** Extracted Nucleic Acid (e.g. 'High Pure PCR Template Preparation Kit').
- **Positive Controls:** Every ModularDx kit is delivered with a single target Positive Control. These controls must be used in single reactions only. Mixtures of controls has been not tested.
- **Premixed Controls:** Alternatively use the Positive Control 90-0600 containing 2 vials, Mix I for targets detected in channels 500, 580 and 640 and Mix II channels 530, 610 (optional 660).
- **Negative Controls:** Use an extracted negative sample containing the Control Reaction target. Alternatively PBS containing the Control target can be used as a negative control.

### 13.3.1. Preparation of Parameter-Specific Reagents (PSR):

**Add 50 µl** PCR-grade water to each reagent vial, mix the solution (invert) and spin down.
For robotic pipetting the resuspension volume can be extended to 55 µl (signals will decrease by 10-20%).

►**Use 0.5 µl** reagent **per 20 µl** reaction.

### 13.3.2. Preparation of the Positive Control

Use Premixed Controls Mix I and II or single Positive Controls. One reagent vial contains for 32 reactions.

**Add 160 µl** PCR-grade water to the vial with the **black** cap. Mix by pipetting up and down 10 times: mixing by vortex may generate aerosol causing contamination.

►**Use 5 µl** positive control **per 20 µl** reaction.

### 13.3.3. Preparation of the Reaction Mix

The table below describes master mix preparation for duplex to hexaplex reactions of 20 µl total volume. Choose between PhHV (**blue**) and RPC (**purple**).

Include one Positive Control (for each target) and at least one 'No Template Control' (NTC) in every run. Premixed controls mix I is negative for pathogens included in mix II and vice versa.

In a cooled tube, prepare the reaction mix by multiplying the single reaction volumes (left column) by the number of reactions including the controls plus one additional reaction; the smallest recommended pipetting volume is 1 µl; prepare a minimum of 10 reactions. Instructions for 96 well plates right column:

| One reaction | Hexaplex Reaction Roche Multiplex Master | 100 reactions |
|---|---|---|
| 6.7 µl | **Water**, PCR-grade (colorless cap, provided with the Roche Master kit) | 670 µl |
| 0.2 µl | **BSA**, 20 µg/µl solution (optional) or **Water** | 20 µl |
| 0.5 µl | **EAV Control Reaction** mix or **Water** | 50 µl |
| 1.0 µl | **Water** or **RPC detection assay** (Roche vial 5) | 100 µl |
| 0.5 µl | **Reagent** mix (primers / probes) first target assay | 50 µl |
| 0.5 µl | **Reagent** mix second target assay or **Water** | 50 µl |
| 0.5 µl | **Reagent** mix third target assay or **Water** | 50 µl |
| 0.5 µl | **Reagent** mix fourth target assay or **Water** | 50 µl |
| 0.5 µl | **Reagent** mix fifth target assay or **Water** | 50 µl |
| 4.0 µl | **Roche Master** (see Roche manual) | 400 µl |
| 0.1 µl | **RT Enzyme** | 10 µl |
| **15.0 µl** | **Volume of Reaction Mix** | **1500 µl** |

| One reaction | Duplex Reaction Multiplex DNA Master (example) | 100 reactions |
|---|---|---|
| 9.9 µl | **Water**, PCR-grade (colorless cap, provided with the Roche Master kit) | 990 µl |
| 0.5 µl | **EAV Control Reaction** mix | 50 µl |
| 0.5 µl | **Reagent** mix (primers / probes) first target assay | 50 µl |
| 4.0 µl | **Roche Master** (see Roche manual) | 400 µl |
| 0.1 µl | **RT Enzyme** | 10 µl |
| **15.0 µl** | **Volume of Reaction Mix** | **1500 µl** |

Mix gently, spin down and **transfer 15 µl** of master mix to each well in a cooled (4°C to 10°C) PCR plate.

**Add 5 µl** of sample or control DNA to each well. Seal plate and centrifuge for 2 min at 1500 × g. **Start run**

Do not touch the sealing foil without gloves. Avoid prolonged waiting periods before starting the run.

 © 2017 TIB MOLBIOL 

# 14. Reading the Results

Perform data analysis as described in the amplification instruments operator's manual. Use the Second Derivative Maximum method (Automated (F'' max). The cycle number, Crossing Point (Cp) value of each sample is calculated automatically. The instrument software tentatively calls results as **Positive (Red plot)**, **Negative (Green plot)**, or **Uncertain (Blue plot)** in the amplification plotview. Repeat analysis for each dye channel used: **(1)** Open a new analysis, **(2)** Name the analysis after the channel and pathogen, **(3)** Apply the color compensation file, **(4)** Analyze and save.

Runs are valid if the results generated for all controls are correct: **Positive Controls** are positive (within Cp range see CoA), **Negative Controls** are negative, and **Control Reactions** (where applicable) are positive.

## 14.1. Control Reaction: Validation of the entire run

Start the analysis in the 660 Channel. Check for amplification curves and verify Cp range.
If the Control Reaction signals are absent the run is not valid and results must not be used.

- If using the premixed Positive Controls and PhHV an amplification curve should be visible for Mix **II**.
- If using the single pathogen Positive Controls and PhHV check the extracted Negative Control for the presence of an amplification curve with a Cp value in the range of 27-33.
- If using the Roche Process Control check that the extracted negative sample containing the Roche Control shows an amplification curve with a Cp range as outlined in the Roche control kit manual.

**Note**: In case that the Negative Control used does not contain the Internal Control Target (not recommended), any 660 channel signals in (pathogen negative) samples may be used to verify the general functionality of the Control Reaction.

## 14.2. Pathogen Results

### 14.2.1. Verification of channel specific results

Perform analyses and review for each channel used, verify that each channel result is valid:

- Target specific ***Negative Control must be negative.***
- Target specific ***Positive Control must be positive*** and within the Cp range detailed in the CoA.
- Target specific Positive Controls for other channels in use must be negative, indicating that the color compensation is switched on and is functional.

### 14.2.2. Target positive and target Negative Sample Results

Identify samples as target-positive, negative (below detection limit) or equivocal :

- Select **'Positive'** results.
- Identify all samples with an amplification curve with a Cp value within the defined cut-off.
- Identify samples with erroneous false-positive calls by visual inspection and correct; false-positive calls can be generated as a result of incorrect interpretation of curves due to baseline or residual signals from another channel that has been not corrected by the color compensation (check for sample positivity in parallel channels).
- Document all **'Positive'** samples and identify samples with manually corrected results.
  For samples with high positive results (Cp < 24) for one target and for positive samples with a negative Control Reaction result; it is recommended to repeat the test with all other targets in single assays to minimize the potential of not detecting low levels of pathogen in mixed infection samples.
- List all samples with a Cp value higher than the cut-off as '**Equivocal'**.
  The cut-off in the CoA is based on the established limit-of-detection (LOD) of each respective assay. The LOD describes the amount of target that will be reliably detected in every run. Results with a later cycle are most likely true positive results, however they are outside the reporting range. It is highly recommended to repeat the test for '**Equivocal'** samples, preferably using a new extract from the same or newly collected specimen. If inhibition is not an issue, use more sample. If the test is not repeated, report **'Below detection limit'**.
- Select **'Negative'** results.
- Visually inspect the negative curves, identify any amplification curves that have been called falsely negative, correct the result and mark samples where the results have been manually corrected. False negative calls are rare.
- Determine **'Negative'** sample results in relation to the Control Reaction results (see 14.2.3)

### 14.2.3. Interpreting true Negative Results

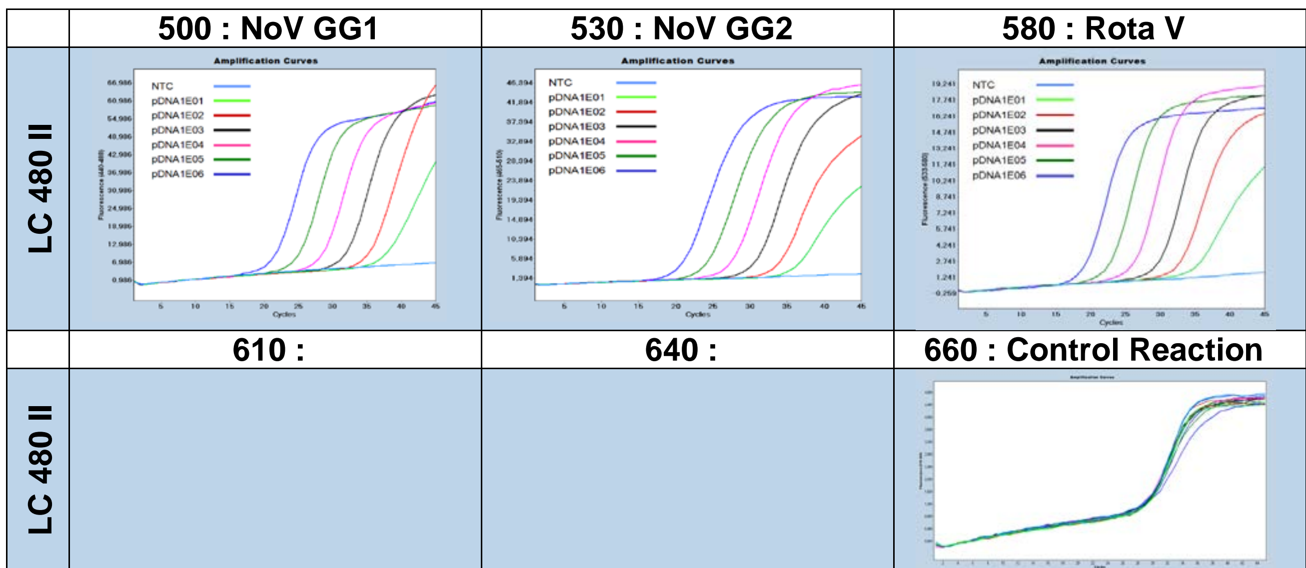
- Identify samples which are negative for all pathogen target channels (no visible amplification curves).
- Verify that these samples have an amplification curve present in the Control Reaction channel (660).
- Report **'Negative'** samples with a positive result for the Control Reaction as **'Not detected'** ( **'target below the defined detection limit')** for the target pathogen.
- Report **'Negative'** samples with a positive result for any other pathogen as **'Not detected'**.
- Report **'Negative'** samples with a negative result for the Control Reaction as "**Inhibited / failed to generate a result**" and request a new sample if applicable.

### 14.2.4. Reporting of Equivocal sample results

If repeat analysis of samples generate amplification curves within the '**Equivocal'** range and samples are not resolved as **'Positive'** or **'Not detected', then** report the sample result as **'Potentially positive, below detection limit'**.

Document all manually corrected results and include the reason for adjustment.

### 14.2.5. Sample Results




## 14.3. Sample Results Interpretation :

For a valid result the Internal Control Reaction (channel 660) must be included in all wells and generate a result within range (Positive or Pass), if the amplification curve is negative or delayed the sample is inhibited: repeat sample processing from extraction. If the NTC generates a positive result in the target channels a contamination might be present: DO NOT report results, repeat entire amplification run.

| 500 | 530 | 580 | 610 | 640 | 660 Control | 500-640 NTC | Result |
|---|---|---|---|---|---|---|---|
| negative | negative | negative | not used | not used | Detectable | negative | No virus detectable |
| Cp < cutoff | negative | negative | not used | not used | irrelevant* | negative | *Pathogen 500 positive* |
| negative | Cp < cutoff | negative | not used | not used | irrelevant* | negative | *Norovirus- positive* |
| negative | negative | Cp < cutoff | not used | not used | irrelevant* | negative | *Rotavirus A positive* |
| negative | negative | negative | not used | not used | irrelevant* | negative | *Pathogen 610 positive* |
| negative | negative | negative | not used | not used | irrelevant* | negative | *Pathogen 460 positive* |
| negative | negative | negative | not used | not used | negative | irrelevant | Inhibition/failure Repeat |
| irrelevant | irrelevant | irrelevant | not used | not used | irrelevant | positive | Contamination Repeat |

* Highly positive samples (Cp<24) may mask the amplification of the internal control reaction and potentially other targets present in the sample (see 14.2.2).

## 15. Assay Limitations

All results should be interpreted by a trained professional in conjunction with the patient history, clinical signs and symptoms, and epidemiological risk factors. Even though low positive results suggest the presence of the respective organism, this organism may not be the cause of the clinical symptoms. Interpretation of results generated by this test should take into consideration the possibility of false results. Negative results do not exclude the presence of the respective pathogen. Molecular test results should not be the sole basis of a patient treatment/management or public health decision.

False positive results may occur from cross-contamination of the target organisms, nucleic acids or amplified product. Improper collection, storage, or transport of specimens may lead to false negative results. Failure to follow the assay procedures may lead to false negative results. Inhibitors present in the samples may lead to false negative results. Potential mutations within the target regions covered by the primer and/or probes of the assay may result in failure to detect the presence of the pathogen.

The test is not validated as a quantitative test for treatment monitoring.

## 16. References


**8.3**

Handbook of Nucleic Acid Purification. Ed. Dongyou Liu (2009)

Utility of the MagNA Pure 96 System …. Roche MagNA Pure System Application Note No. 6 (2015)

Universal extraction method for gastrointestinal pathogens. Halstead et al., 2013

**9.1 Norovirus**

Evaluation of var. real-time RT-PCR assays for detection and quantitation of human norovirus. Butot et al., (2010)

Detection and characterization of norovirus outbreaks in Germany: application of a one-tube RT-PCR using a fluorogenic real-time detection system. Höhne et al., 2004

**9.2 Rotavirus**

The development, implementation and evaluation of a Real-Time PCR-based diagnostic service for viral causes of infectious intestinal disease. Gunson, 2007

## 17. Certificate of Origin

Product is not from human, animal or plant origin. Country of Origin: Germany

## 18. Contents and Material Safety Data (MSDS)

Product contains :
99.8% Synthetic oligonucleotides (< 100 microgramm)
0.1% CAS 77-86-1 Tris (hydroxymethyl) aminomethane
0.1% CAS 60-00-4 Ethylenediamine tetraacetic acid (EDTA)

Product is not hazardous, not toxic, not IATA-restricted.

According to OSHA 29CFR1910.1200, Australia [NOHSC:1005, 1008 (1999)] and the EU Directives 67/548/EC and 1999/45/EC any products which do not contain more than 1% of a component classified as hazardous or classified as carcinogenic do not require a Material Safety Data Sheet (MSDS).

Customs Tarif no.(HS code) 2934 9990

## 19. Version History

Notes in red mark: events requiring changes in procedures.

| V160202 | Release version | 2016-04-20 |
|---|---|---|
| V170717 | 8.3 Extraction; 9. Pathogen information, 13.3.3 BSA | 2017-07-17 |

## 20. Manufacturer and Contact Details

Report IVD device observations, deviations and problems including lot number(s) and a brief error description to service@tib-molbiol.de and your local Roche representative

**TIB MOLBIOL** Syntheselabor GmbH | Eresburgstr. 22-23 | D-12103 Berlin | Germany
Tel. +49 30 78 79 94 55 | FAX +49 78 79 94 99 | dna@tib-molbiol.de | WWW.TIB-MOLBIOL.COM
Geschäftsführer (CEO): Olfert Landt | Register HRB 93163 B | Registergericht Berlin Charlottenburg

Distributed by Roche - www.lifescience.roche.com

**Notice to Purchaser – Patents and Trademarks**

The purchase of the present product grants the right to use it in order to perform the amplification and detection of nucleic acid sequences for in-vitro diagnostic purpose on human origin samples. No other kind of license is transferred except the right to use the present product derived from its purchase. Other than expressly stated licenses, TIB MOLBIOL makes no warranty that this kit and/or its use(s) do not infringe the rights of third-parties.

LIGHTMIX is a trademark of TIB MOLBIOL. LIGHTCYCLER, MAGNA PURE, HIGH PURE and COBAS Z are trademarks of Roche.
All other product names and trademarks are the property of their respective owners.