In-vitro diagnostic reagent instructions to use. For *in vitro* use only

LightMix® Modular Gastro Parasite 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

Order no.	Roche no.	Name / Target		channel
50-0611-96	07979746001	*E.histolytica*	CE-IVD	500
53-0612-96	07979754001	*Giardia*	CE-IVD	530
58-0613-96	07989342001	*Dientamoeba fragilis*	CE-IVD	580
61-0614-96	07989334001	*Cryptosporidium*	CE-IVD	610
64-0615-96	07989326001	*Blastocystis*	CE-IVD	640
66-0901-96	07093802001	PhHV	Extraction Control	660
90-0600-01		Gastro Pos Control		n.a.

4. Multiplex PCR Combinations (example)

Gastro Parasite Multiplex PCR

Color Compensation 40-0320 is mandatory for Multiplex PCR

500	530	580	610	640	660
	Giardia				Control: PhHV or RPC*
	Giardia		Cryptosporidium		
E.histolytica	Giardia		Cryptosporidium		
E.histolytica	Giardia	Dientamoeba	Cryptosporidium		
E.histolytica	Giardia	Dientamoeba	Cryptosporidium	Blastocystis	

480 II	z 480	
X	X	Duplex
X	X	Triplex
X		4plex
X		5plex
X		6plex

* Roche RNA Process Control (RPC) must not be used in combination with the *Dientamoeba* assay. Using the RPC requires to include the reverse transcription step, which may cause interferences. Development and evaluation studies have been performed with PhHV.

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 and Stability

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 post 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 DNA Master		Cat.-No. 07 339 585 001
LightCycler® Multiplex RNA Virus Master	(optional)	Cat.-No. 06 754 155 001
RNA Process Control Kit	(optional)	Cat.-No. 07 099 592 001
Uracil-DNA Glycosylase (UNG)	(optional)	Cat.-No. 03 539 806 001
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 an increased number positive results as compared to 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 of the 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.

Each kit contains primers designed to specifically amplify one or more short fragments within 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 2°C to 8°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 2°C to 8°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 or Roche RPC; not tested: EAV) to a final sample volume of 200 µl in the 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. E. histolytica

Entameoba histolytica infections may be asymptomatic. Symptomatic presentations include mild diarrhea, however in more severe cases patients can present with dysentery (fever, bloody diarrhea) and whilst very rare extraintestinal disease (e.g. liver abscess).

A 90 bp long fragment from the rRNA gene is amplified and detected with a Cyan500 labeled probe.

Specificity: The assay detects specifically *E. histolytica* and excluding *E.dispar*. Testing of other pathogens that may be present in stool samples generated not detectable results (refer to table 9.6).

Sensitivity.The limit of detection (LOD Probit-95) obtained with clinical samples is 2.5 copies per reaction. Sensitivity in the presence of 100,000 copies of another target (Cp = 24) from this panel is at least 10 copies.

9.2. Giardia

Giardia spp. infections may be asymptomatic; or present with diarrhea, cramping, foul smelling greasy stools, nausea and weight loss.

A 62 bp long fragment from the 18S rRNA gene is amplified and detected with a FAM labeled hydrolysis probe.

Specificity: The assay will detect *G. intestinalis* (*lamblia*), *G. duodenalis*, most *G. microti* but may not detect *G. ardeae, G. muris* and *G. psittaci*. Testing of other pathogens that may be present in stool samples generated not detectable results (refer to table 9.6).

Sensitivity.The limit of detection (LOD Probit-95) obtained with clinical samples is 3.6 copies per reaction. Sensitivity in the presence of 100,000 copies of another target (Cp = 24) from this panel is at least 10 copies.

9.3. Dientamoeba

Dientamoeba fragilis can be found in patients with non acute diarrhea or patient with abdominal cramping, diarrhea, vomiting and decreased appetite. Presentation with a peripheral blood eosinophilia is rare.

A 93 bp long fragment from the 5.8S rRNA gene is amplified with specific primers and detected with a R6G labeled hydrolysis probe.

Specificity: Testing of other pathogens that may be present in stool samples generated not detectable results (refer to table 9.6).

Sensitivity.The limit of detection (LOD Probit-95) obtained with clinical samples is 6.2 copies per reaction. Sensitivity in the presence of 100,000 copies of another target (Cp = 24) from this panel is at least 10 copies.

9.4. Cryptosporidium

Cryptosporidium spp can cause mild or severe diarrhea, cramping, and low grade fevers. Patients who are immunocompromised are at an increased risk of severe disease.

Two 73 and 118 bp long fragments from the Oocyst Wall Protein (COWP) gene are amplified with specific primers and detected with LC610 labeled hydrolysis probes.

Specificity. According to *in-silico* analysis this kit will detect *C. hominis, parvum, meleagridis, tyzzeri, wrai, erinace, cuniculus, ferret*, and *viatorum* but will not detect *C. ubiquitum*. Testing of other pathogens that may be present in stool samples generated not detectable results (refer to table 9.6).

Sensitivity.The limit of detection (LOD Probit-95) obtained with clinical samples is 3.2 copies per reaction. Sensitivity in the presence of 100,000 copies of another target (Cp = 24) from this panel is at least 10 copies.

9.5. Blastocystis

Blastocystis spp infections can be asymptomatic. Patients may present with watery diarrhea and cramping. Patients are usually afebrile.

A 148 bp long fragment from the ribosomal 18S RNA gene is amplified with specific primers and detected with a LC640 labeled hydrolysis probe.

Specificity. Testing of other pathogens that may be present in stool samples generated not detectable results (refer to table 9.6).

Sensitivity.The limit of detection (LOD Probit-95) obtained with clinical samples is 2.4 copies per reaction. Sensitivity in the presence of 100,000 copies of another target (Cp = 24) from this panel is at least 10 copies.

9.6. Summary Analytical Specificity / Cross-Reactivity

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

10. Product Specification - Certificate of Analysis

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

Lot-specific **signal levels** (normalized to a manufacturer internal reference, tested in a single target PCR), lot-specific **Cp value ranges** (from diluted positive controls) and derived **cut-off Cp values** (predicted Cp for ~5 copies per reaction) are printed in the lot-specific Certificate-of-Analysis included with the product.

11. Evaluation Studies

11.1 *E.histolytica*

In a clinical study with 744 diarrhea fecal samples the prevalence for *Entamoeba spp* (kit 50-0610) was 4%. Sensitivity and specificity of this assay as part of a hexaplex test was 100%; PPV and NPV were 100%.

A study on 526 samples performed in Australia (kit 50-0611) showed two positive PCR results confirmed by an alternative PCR method but not by microscopy; using microscopy as the gold standard both the specificity and NPV were 100%.

11.2 *Giardia*

In a clinical study including 744 diarrhea fecal samples the prevalence for *Giardia* was 2.9%. Sensitivity of this assay run in a hexaplex PCR reaction was 97.7%, specificity 94.6%, PPV 79% and the NPV was 99.5%.

A second study performed in Australia included 526 samples. The prevalence was reported to be 12 %. Compared to microscopy as the gold standard the sensitivity was 100%, specificity 98.5%, NPV 100%, and the PPV was 90.1%. Notably, all positive PCR results were confimed with an alternative PCR assay.

11.3 *Dientamoeba*

In a clinical study including 744 diarrhea fecal samples the prevalence for *Dientamoeba* was 9.2%. The sensitivity in hexaplex PCR was 100%, the specificity was 87.5%, the PPV 45% and the NPV 100%.

A second study performed in Australia included 526 samples. The prevalence was 1.7 %. The PCR sensitivity was 100%, specificity was 86,3%, NPV was 100%, while the PPV was 11%. Some 20% of the discordant results could be confirmed with an alternative PCR assay.

11.4 *Cryptosporidium*

In a clinical study including 744 diarrhea fecal samples the prevalence for *Cryptosporidium* was 1.9%. Sensitivity and specificity of the assay performed in a hexaplex PCR were 100%, PPV and NPV were 100%.

A second study performed in Australia included 526 samples. The prevalence was reported to be 5.5 %. PCR sensitivity was 100%, specificity was 99.9%, NPV was 100%, and the PPV was 93.5%. Notably, all dicordant PCR results were positive using an alternative PCR assay.

11.5 *Blastocystis*

In a clinical study including 744 diarrhea fecal samples the prevalence for *Blastocystis* was 17.7%. Sensitivity of this assay as part of a hexaplex test was 97.7%, the specificity was 94.6%, PPV 79% and the NPV was 99.5%.

A second study performed in Australia included 526 samples. The prevalence was reported to be 16%. Compared to microscopy PCR sensitivity was 100%, specificity 93.9%, NPV 100%, and the PPV was 75.5%. Notably, all dicordant PCR results were positive using an alternative PCR assay.

12. Evaluation Data Summary

Channel	500	530	580	610	640
Pathogen	**E.histolytica**	**Giardia**	**Dientamoeba**	**Cryptosporidium**	**Blastocystis**
LOD^{single} (copies/rx)	n.d.	n.d.	n.d.	n.d.	n.d.
$LOD^{multiplex}$ (copies/rx)	2.6	3.1	5.7	3.2	2.3
95% CI	1.6 - 3.2	2.1 - 8.6	4.1 - 56.7	2.8 - 3.8	1.6 - 51.7
Specificity	passed	passed	passed	passed	passed
Diag. Sensitivity	100%	97.7%	100%	100%	97.7%
Diag. Specificity	100%	94.6%	87.5%	100%	94.6%
Prevalence	4.0%	2.9%	9.2%	1.9%	17.7%
PPV	100%	79%	45%	100%	79%
NPV	100%	99.5%	100%	100%	99.5%
Intra Assay Variation	0.42%	0.14%	0.97%	0.24%	0.56%
Inter Assay Variation	0.83%	0.25%	0.96%	0.38%	0.38%
Inter Lot Variation	0.61%	0.31%	1.27%	0.44%	0.49%

n.d. = not determined LOD values for 10 µl reactions with 5 µl sample in 384 well plates were in the same range.

13. Instructions for Use

See the Instrument operator's manual for details. Program instruments prior to reagent preparation.

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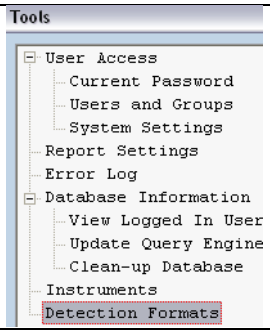
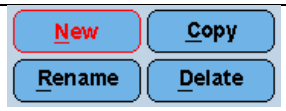
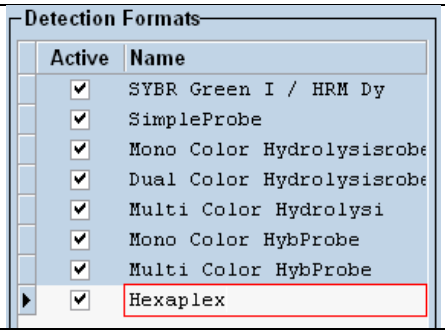
13.1. Dye Channels and Color Compensation

1) Open Tools	2) Select Detection Formats	3) New	4) Set name Hexaplex
			

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 instruction 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	558-610	558-640	615-670
LightCycler® 480 II	440-488	465-510	533-580	533-610	533-640	618-660
cobas z 480 analyzer	465-510	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

* Instrument settings may be changed but require verification by the operator.

13.2. Programming Roche '480' Instruments

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

The protocol consists of three program steps:

- 1: Denaturation: sample denaturation and enzyme activation
- 2: Cycling: PCR-amplification
- 3: Cooling: cooling the instrument

Program Step:	*UNG*[1]	*RT Step*[2]	***Denaturation***	***Cycling***			***Cooling***
Parameter							
Analysis Mode	***None***	***None***	None	Quantification mode			None
Cycles	*1*	*1*	1	45			1
Target [°C]	40	55	95	95	60	72	40
Hold [hh:mm:ss]	00:10:00	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	4.4	2.2	4.4	1.5
Ramp Rate [°C/s] **384**	4.6	4.6	4.6	4.6	2.4	4.6	2.0
Acquisition Mode	None	None	None	None	**Single**	None	None

[1]*optional if UNG is included* [2]*optional if combined with 1-Step RT-PCR.*

UNG and LC Multiplex RNA Virus Master cannot not be combined in a single reaction.

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. Mixing 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 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 that can lead to 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**). Use of **UNG** and **RT enzyme** are mutually exclusive.

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 complete plates right column:

One reaction	Pipetting Options Table up to Hexaplex Reaction	100 reactions
6.5 µl	**Water**, PCR-grade (Roche kit, colorless cap)	650 µl
0.2 µl	**BSA**, 20 µg/µl solution (optional) or **Water**	20 µl
0.5 µl	**PhHV 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 (primer / 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 Multiplex Master** (see Roche manual)	400 µl
0.2 µl	**UNG** (for use with DNA Master only) or **Water**	20 µl
0.1 µl	**RT Enzyme** (if combined with **RNA**) or **Water**	10 µl
15.0 µl	**Volume of Reaction Mix**	**1500 µl**

One reaction	Duplex Reaction Multiplex DNA Master (example)	100 reactions
9.8 µl	**Water**, PCR-grade (Roche kit, colorless cap)	980 µl
0.2 µl	**BSA**, 20 µg/µl solution (optional)	20 µl
0.5 µl	**PhHV Control Reaction** mix	50 µl
0.5 µl	**Reagent** mix (primer / probes) target assay	50 µl
4.0 µl	**Roche Multiplex Master** (see Roche manual)	400 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 (2°C to 8°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.

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: If the Negative Control reaction does not contain the Internal Control Target (not recommended) any other 660 channel signals from pathogen-negative samples may be used to verify that the Control Reaction is working.

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, not detected (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 a 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 samples with a mixed infection.
- 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 threshold 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, extract a higher amount of sample. If the test is not repeated, report **'Below detection limit'**.
- Select **'Not detected'** 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 **'Not detected'** 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',** 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

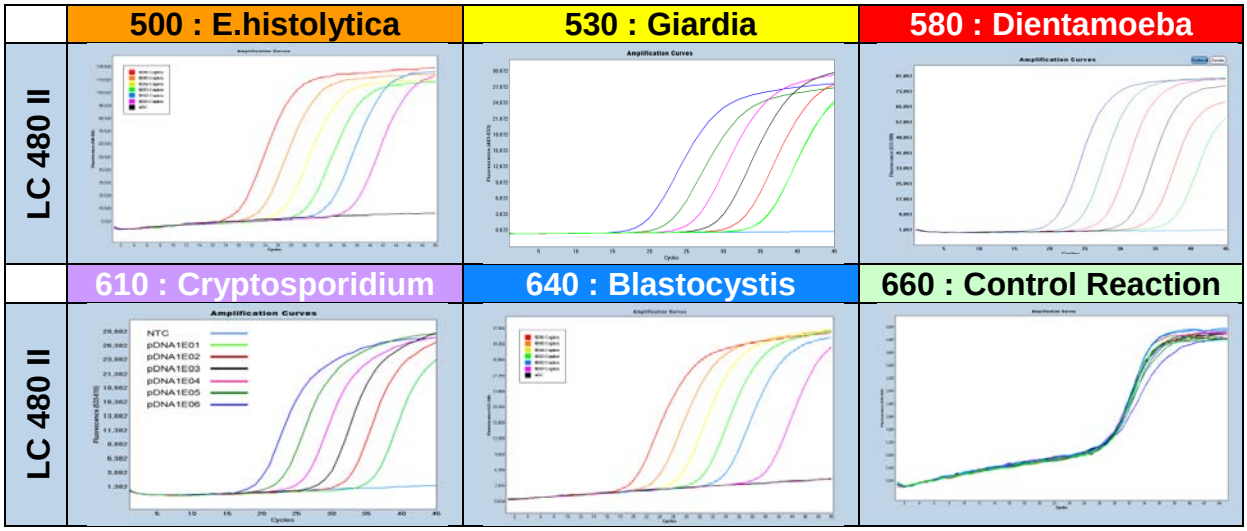


Fig. Sample Results obtained with plasmid standard dilutions in a single PCR experiment.

14.3. Sample Result Interpretation:

The Control Reaction (660) is to be included in every reaction and must generate for the Negative Control and in the pathogen-negative wells a result within range (first line); if negative or massively delayed (line 7) the sample is inhibited: Repeat sample processing from extraction. A positive NTC result in a target channels (line 8) identifies contamination: DO NOT report results, repeat the entire amplification run.

500	530	580	610	640	660 Control	500-640 NTCs	Result
negative	negative	negative	negative	negative	in range (27-33)	negative	None of the tested pathogen detectable
Cp < cutoff	negative	negative	negative	negative	irrelevant*	negative	*E.histolytica*
negative	Cp < cutoff	negative	negative	negative	irrelevant*	negative	*Giardia spp.*
negative	negative	Cp < cutoff	negative	negative	irrelevant*	negative	*Dientamoeba fragilis*
negative	negative	negative	Cp < cutoff	negative	irrelevant*	negative	*Cryptosporidium spp.*
negative	negative	negative	negative	Cp < cutoff	irrelevant*	negative	*Blastocystis spp.*
negative	negative	negative	negative	negative	not detectable	irrelevant	Inhibition/failure Repeat
irrelevant	irrelevant	irrelevant	irrelevant	irrelevant	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

Since the PPV is reported to be less than 90% for some assays, it is recommended that PCR-positive results are verified by microscopy and or culture.

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 in the event of 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
Real-time PCR for detection and differentiation of Entamoeba histolytica and Entamoeba dispar in fecal samples. Blessmann et al., 2002

9.2
Simultaneous detection of E. histolytica, G. lamblia, and C. parvum in fecal samples by using multiplex rt PCR. Verwei et al., 2004

Multiplex rt PCR assay for detection of E. histolytica, G. intestinalis, and Cryptosporidium spp. Haque et al., 2007

9.3
Detect Real-time PCR for the detection of Dientamoeba fragilis in fecal samples. Verwei et al., 2007

9.4
Multiplex rt PCR assay for detection of E. histolytica, G. intestinalis, and Cryptosporidium spp. Haque et al., 2007

9.5
Development and evaluation of a genus-specific, probe-based, internal process controlled real-time 6 PCR assay for sensitive and specific detection of Blastocystis. Stensvold et al., 2012

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
V161122	Revised version for product inserts V161122	2016-11-22
V170717	8.3 Extraction, 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.

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CE IVD

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