Instructions For Use

LightMix® Modular *Necator americanus*

500

Cat.-No. 50-0714-96 Roche SAP n° 00 000 000 000

Kit with reagents for 96 PCR reactions 20 µl for detection of N. americanus [lyophilized]

1. Content Storage and Expiry

Storage at Arrival:

1 Vial orange 96 reactions N.americanus (lyophilized)
1 Vial black cap Positive Control (32,000 copies, lyophilized)

Store cooled or at ambient temperature
Do not freeze the lyophilized reagents.

- Lyophilized kits are stable for at least 6 months (4°C to 25°C in the dark). See lot-specific expiry date.
- Dissolved reagents are stable for at least 2 weeks if stored protected from light and cooled (4°C).
- Dissolved reagents can be stored long-term at -20°C (within expiry). Avoid multiple freeze-thaw cycles.
- Dissolved positive controls must be stored at -20°C. Avoid multiple freeze-thaw cycles.

2. Additional Reagents required

LightCycler® Multiplex DNA Master Cat.-No. 07 339 585 001

3. Introduction

Infectious diarrhea can be caused by viruses, bacteria or parasites. The nematode *N. americanus* has a worldwide distribution, though primarily in tropical regions. Together with *Ancylostoma duodenale* it is the most common human intestinal helminth and responsible for the hookworm disease. During pregnancy hookworm infections can cause retarded growth of the fetus, premature birth and a low birth weight.

4. Description

A 119 bp long fragment from the 18S RNA gene is amplified with specific primers and detected with a Cyan500 labeled hydrolysis probe.

5. Specification

This assay detects 10 genome equivalent copies or less per reaction (plasmid DNA dilution).

6. Sample Material and Extraction

Typical samples are from feces or eventually rectal swabs. See ModularDx ***Extraction Protocols***.

7. Material Safety Data (MSDS)

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).

Product is not hazardous, not toxic, not IATA-restricted. Product is not from human, animal or plant origin. Product contains synthetic oligonucleotide primers and probes.

8. Instructions for Use

- Instrument programming see document ***ModularDx Programming***
- Colour Compensation see instructions in ***40-0320 Universal Color Compensation Hexaplex***
- Pipetting instructions multiplex PCR see ***ModularDx Multiplex***

8.1. Programming Roche 480 Instruments

See the Instrument operator's manual for details. Start programming before preparing the solutions. The protocol consists of three program steps:

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

Detection Format 500 Channel **Set Quant Factor 10, Max Integration time 1 sec**
LightCycler® 480 Instrument: 450-500
LightCycler® 480 II Instrument: 440-488
cobas z 480 Analyzer (open channel): No filter combination for Cyan500 (opt. use FAM channel)

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

* *optional to combine with 1-Step RT-PCR*

Table 1

8.2. Experimental Protocol

- **Sample material:** Use aqueous nucleic acid preparations (e.g. 'High Pure PCR Template Preparation Kit').
- **Negative control:** Always run at least one no-template control (NTC) - replace the template NA with water.
- **Positive control:** Run a positive control - replace the template NA with the provided Positive Control.

For an increased sensitivity use 10 µl sample per 20 µl reaction, in case that inhibition is likely to occur, e.g. extracts obtained from fecal samples, use 5 µl. For 10 µl reactions in 384 well plates use 5 µl /2.5 µl.

8.2.1. Preparation of Parameter-Specific Reagents (PSR, 96 reactions):

One reagent vial with an orange cap contains all primers and probe to run 96+ LightCycler® reactions.

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

►**Use 0.5 µl** reagent for a 20 µl PCR reaction.

8.2.2. Preparation of the Positive Control

Add 160 µl RNase/DNase-free Tris buffer or water to the vial with the black cap, for 10 µl sample add **320 µl**. Mix the solution by pipetting up and down 10 times. If vortexing spin down to collect the solution.

Notes: Opening of this vial may cause contaminations of the work-space (aerosol). Use of Tris buffer pH 8.0-8.5 increases the long-term stability in solution. Store dissolved controls frozen.

►**Use 5 µl** positive control (≈ 1,000 copies) for a 20 µl PCR reaction (or 10 µl if using 10 µl sample).

8.2.3. Preparation of the Reaction Mix

Multiply volumes by the number of reactions plus controls and one reserve and prepare in a cooled tube:

For use with the Roche LightCycler® Multiplex DNA Master		
for 5 µl extract	**Component**	**10 µl extract**
10.5 µl	**Water**, PCR-grade (colorless cap, provided with the Roche Master kit)	5.5 µl
0.5 µl	**Reagent** mix (parameter specific reagents containing primers and probes)	0.5 µl
--	Control Reaction and additional assays (Multiplex PCR)	--
4.0 µl	**Roche Master** (see Roche manual)	4.0 µl
15.0 µl	**Volume of Reaction Mix**	**10.0 µl**

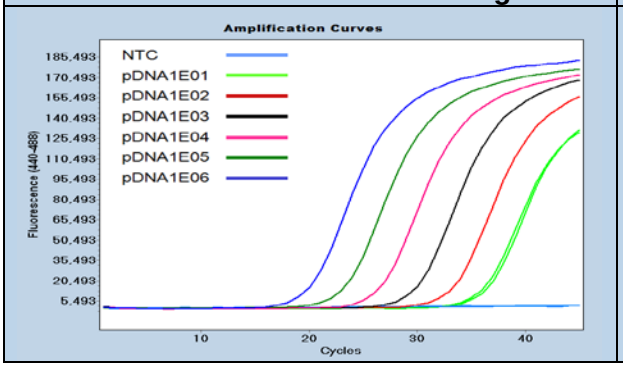
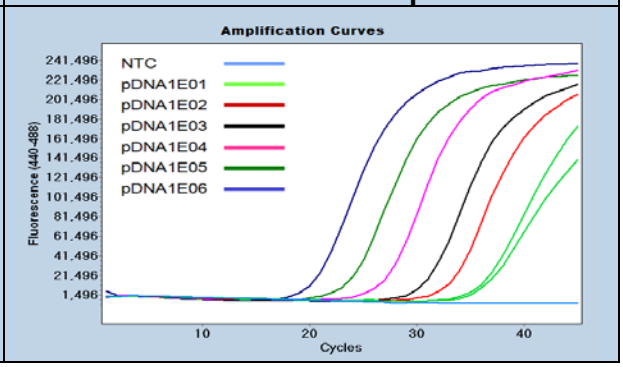
Table 2

Mix gently, spin down and **transfer 15 µl (10 µl)** per well.

Add 5 µl (10 µl) of sample or control to each well for a final reaction volume of 20 µl. Seal plate and centrifuge.

Start run

9. Typical Results (Data from LightCycler® 480 II system)

Channel 500 : N.americanus – Single PCR	Data obtained in Multiplex PCR
	

Dilution row 1E6 to 10 copies / reaction

Figure 1

10. Reading the Results

Perform data analysis as described in the operator's manual. For multiplex assays select the color compensation. We recommend using the Second Derivative Maximum method (Automated (F" max). View results in the 500 channel. The negative control (NTC) must show no signal.

Channel 500 (sample)	Channel 660 Control Reaction	Channel 500 NTC Control	Result
No amplification	**Detectable**	**Negative**	**Not detectable**
Amplification Cp < 37 +	Not relevant	**Negative**	**N.americanus Positive**
No amplification	**Not detectable**	Not relevant	**PCR failure Repeat**
Amplification signal	Not relevant	**Positive**	**Contamination Repeat**

Notes: cobas z 480 Analyzer signal levels are about 50% compared to LightCycler® 480 II results.
+ Recommendation : Define the cut-off 2-4 cycles higher than observed for 10 copies

11. References

Simultaneous Detection and Quantification of Ancylostoma duodenale, Necator americanus, and Oesophagostomum bifurcum in Fecal Samples Using Multiplex Real-Time PCR. Verweij, Brienen, Ziem, Yelifari, Polderman, and Van Lieshout. Am. J. Trop. Med. Hyg., 77(4), 2007, pp. 685–690.

12. Multiplex PCR Compatibility (Gastro Panel)

This assay can be combined with other assays up to 6plex reactions including an internal control (IC) or a spiked extraction control (for example PhHV) as depicted below :

Gastro Multiplex PCR and Instrument Compatibility

Color Compensation 40-0320 is mandatory for Multiplex PCR

500	530	580	610	640	660
N.americanus					
N.americanus		S. stercoralis	A.lumbricoides		
N.americanus		S. stercoralis	A.lumbricoides	A.duodenale	
N.americanus		S. stercoralis	A.lumbricoides	H.nana	PhHV
N.americanus	Cyclospora	S. stercoralis	A.lumbricoides	A.duodenale	
N.americanus	Cyclospora	S. stercoralis	A.lumbricoides	H.nana	

480 II	z 480	LC96	LC2.0	Nano
X	X	X		
X	X	X		
X	X			
X	X			
X				
X				

Table 3

This kit shares the 500 channel with 50-0705-96 Enterobius vermicularis.

13. Version History

V160313	Release version	2016-07-10

Certificate of Analysis (CoA)

Lot n°
Expiry :

TIB MOLBIOL

Dilution	1E6	1E5	1E4	PC	1E2	1E1	passed
Cp range				29-31			
Measured							
Signal level				80-120			
Measured							
Negatives	10/10						✓

***Note**:* Cp (crossing point) values collected with pDNA (single target PCR). Fluorescence (FL) levels depend on instrument settings and may vary. The Cp values will vary from instrument to instrument by up to 2 cycles, while the distance between two dilution steps should be relative constant (ΔCp).

QC Acceptance Date:

We, the undersigned, certify that the product designated above has been obtained in accordance with the rules of production and quality control.

Name(s) :

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