RIDA®GENE Clostridium difficile

REF PG0835

R-Biopharm AG, An der neuen Bergstraße 17, 64297 Darmstadt, Germany
Phone: +49 (0) 61 51 81 02-0 / Fax: +49 (0) 61 51 81 02-20

1. Intended use

For *in vitro* diagnostic use. RIDA®GENE Clostridium difficile is a multiplex real-time PCR for the direct, qualitative detection of *Clostridium difficile* (16s-rDNA) and *Clostridium difficile* toxin A (tcdA) / B (tcdB) genes from human stool samples and cultures. The RIDA®GENE Clostridium difficile multiplex real-time PCR can be used as an aid in diagnosis of *Clostridium difficile* associated diarrhea (CDAD).

2. Summary and Explanation of the test

Clostridium difficile, a gram-positive, spore-forming anaerobic bacterium was first described in 1935 by Hall and O'Toole as a component of the intestinal microflora in healthy neonates.[1] In the late 1970s, however, *Clostridium difficile* was identified as the cause of antibiotica-associated diarrhea and pseudomembranous colitis.[2] Today *Clostridium difficile* is one of the most common causes of nosocomial diarrhea. *Clostridium difficile* is responsible for 15 - 25 % of antibiotica-associated diarrhea and nearly all cases of pseudomembranous colitis.[3] The predisposing risk factors for CDAD are for example antibiotica exposure, advanced age as well as number and duration of hospitalization.[4] However, *Clostridium difficile* infection is also seen in an increasing number of non-antibiotic-treated and non-hospitalized individuals.
The symptoms range from mild diarrhea to intestinal infections of variable severity, including pseudomembranous colitis, the most severe form of antibiotic-induced inflammatory bowel disease. Clinically symptomatic cases are caused by toxigenic *Clostridium difficile* strains that produce toxin A and toxin B. In recent years the incidence and severity of *Clostridium difficile* infections increased worldwide.
Real-time PCR permits a rapid, highly sensitive and specific detection of *Clostridium difficile* infection. An early and reliable diagnosis of *Clostridium difficile* infection makes it possible to administer specific treatment of CDAD patients and also to initiate hygiene measures to prevent nosocomial transmission.

3. Test principle

The RIDA®GENE Clostridium difficile multiplex real-time PCR is a molecular diagnostic test for the direct, qualitative detection of *Clostridium difficile* (16s-rDNA) and *Clostridium difficile* toxin genes A (tcdA) / B (tcdB) from human stool samples and cultures.
After DNA-Isolation, amplification of the gene fragments specific for *Clostridium difficile* and toxin A and B of *Clostridium difficile* (if present) occurs. The amplified targets are detected with hydrolysis probes, which are labeled at one end with a quencher and at the other end with a fluorescent reporter dye (fluorophore). In the presence of a target the probes hybridize to the amplicons. During the extension step the Taq-Polymerase breaks the reporter-quencher proximity. The reporter emits a fluorescent signal which is detected by the optical unit of a real-time PCR cycler. The fluorescence signal increases with the amount of formed amplicons. The RIDA®GENE Clostridium difficile assay contains an Internal Control DNA (ICD) as an internal control of sample preparation procedure and/or to determine possible PCR inhibition.

4. Reagents provided

Tab. 1: Reagents provided (Reagents provided in the kit are sufficient for 100 determinations)

Kit Code	Reagent	Amount		Lid Color
1	Reaction Mix	2x	1050 µl	yellow
2	Taq-Polymerase	1x	80 µl	red
D	Internal Control DNA	2x	1700 µl	orange
N	No Template Control	1x	450 µl	white
P	Positive Control	1x	200 µl	blue

5. Storage instructions

- Protect all reagents from light and store at -20 °C. All reagents can be used until the expiration date. After expiry the quality guarantee is no longer valid.
- Carefully thaw reagents before using (e.g. in a refrigerator at 2 - 8 °C).
- Reagents can sustain up to 20 freeze/thaw cycles without influencing the assay performance (e.g. after the first thawing separate it in aliquots and freeze immediately).
- During PCR preparation all the reagents should be stored cold in an appropriate way (2 - 8 °C).

6. Additional necessary reagents and necessary equipment

The RIDA®GENE Clostridium difficile multiplex real-time PCR assay is suitable for use with following extraction platforms and real-time PCR instruments:

Tab. 2: Necessary equipment

Extraction platforms	
R-Biopharm	RIDA® Xtract
Promega	Maxwell® RSC
bioMérieux	NucliSENS® easyMAG™
Real-time PCR instruments	
Roche	LightCycler® 480II
Agilent Technologies	Mx3005P
Applied Biosystems	ABI 7500
Bio-Rad	CFX96™
QIAGEN	Rotor-Gene Q

Note: Only use 0.1 ml tubes on the Rotor-Gene Q (QIAGEN).

If you want to use other extraction platforms or real-time PCR instruments please contact R-Biopharm at mdx@r-biopharm.de.

- RIDA®GENE Color Compensation Kit IV (PG0004) for use with the LightCycler® 480II
- Real-time PCR consumables (plates, tubes, foil)
- Centrifuge with a rotor for the reaction vials
- Vortexer
- Pipettes (0.5 – 20 µl, 20 – 200 µl, 100 – 1000 µl)
- Filter tips
- Powder-free disposal gloves
- PCR water (BioScience grade, nuclease-free, DEPC treated water)

7. Precautions for users

For *in-vitro* diagnostic use.
This test must only be carried out by trained laboratory personnel. The guidelines for working in medical laboratories have to be followed. The instruction manual for the test procedure has to be followed. Do not pipet samples or reagents by mouth. Avoid contact with bruised skin or mucosal membranes. During handling reagents or samples, wear appropriate safety clothing (appropriate gloves, lab coat, safety goggles) and wash your hands after finishing the test procedure. Do not smoke, eat or drink in areas where samples or reagents are being used.

- Extraction, PCR preparation and the PCR run should be separated in different rooms to avoid cross-contaminations.
- Samples must be treated as potentially infectious as well as all reagents and materials being exposed to the samples and have to be handled according to the national safety regulations.
- Do not use the kit after the expiration date.

All reagents and materials used have to be disposed properly after use. Please refer to the relevant national regulations for disposal.

For more details see Safety Data Sheets (SDS) at www.r-biopharm.com.

8. Collection and storage of samples

8.1 Sample preparation from stool samples

For DNA isolation of human stool samples, use a commercially available DNA isolation kit (e.g. RIDA® Xtract (R-Biopharm)) or DNA extraction system (e.g. Maxwell® RSC (Promega)). Extract DNA according to the manufacturer's instructions.
We recommend to dilute the stool samples before extraction 1:3 with water. Vortex the diluted stool sample intensely and centrifuge at 1000 x g for 30 sec. Use from the supernatant the appropriate volume according to the manufacturer's instruction.

The RIDA®GENE Clostridium difficile assay contains an Internal Control DNA that detects PCR inhibition, monitors reagent integrity and confirms that nucleic acid extraction was sufficient. The Internal Control DNA can either be used as PCR inhibition control or as extraction control for the sample preparation procedure and as PCR inhibition control.

If the Internal Control DNA is used only as a PCR inhibition control, 1 µl of the Internal Control DNA should be added to the Master-Mix (see Tab.4).

If the Internal Control DNA is used as an extraction control for the sample preparation procedure **and** as PCR inhibition control, 20 µl of the Internal Control DNA has to be added during extraction procedure. The Internal Control DNA should always be added to the specimen-lysis buffer mixture

and must **not** be added directly to the specimen. We also recommend to add 1 µl of the Internal Control DNA to the negative control and positive control PCR Mix.

8.2 Sample preparation from cultures

For DNA isolation from culture the following procedure is recommended: Add 1 ml PCR water into a preparation tube. Collect colonies with an inoculation loop and suspend them in the prepared PCR water. Cut or break the inoculation loop stem. Cap the preparation tube tightly and vortex strongly for 60 seconds. Heat and shake the preparation tube at 95 °C for 10 min in a heating block. Centrifuge for 1 min at 13.000 x g and apply the supernatant as sample.

Note: Repeat the centrifugation step in case of strong turbidity (if necessary).

The RIDA®GENE Clostridium difficile assay contains an Internal Control DNA that detects PCR inhibition, monitors reagent integrity and confirms that nucleic acid extraction was sufficient. The Internal Control DNA can either be used as PCR inhibition control or as extraction control for the sample preparation procedure and as PCR inhibition control.

If the Internal Control DNA is used only as a PCR inhibition control, 1 µl of the Internal Control DNA should be added to the Master-Mix (see Tab.4).

If the Internal Control DNA is used as an extraction control for the sample preparation procedure **and** as PCR inhibition control, 20 µl of the Internal Control DNA has to be added during extraction procedure. The Internal Control DNA should always be added to the specimen-PCR water mixture and must **not** be added directly to the specimen. We also recommend to add 1 µl of the Internal Control DNA to the negative control and positive control PCR Mix.

9. Test procedure

9.1 Master-Mix preparation

Calculate the total number of PCR reactions (sample and control reactions) needed. One positive control and one negative control must be included in each assay run.

We recommend calculating an additional volume of 10 % to compensate imprecise pipetting (see Tab. 3, Tab. 4). Thaw, mix gently and briefly centrifuge the Reaction Mix, the Taq-Polymerase, the Positive Control, the No Template Control and the Internal Control DNA before using. Keep reagents appropriately cold during working step (2 - 8 °C).

Tab. 3: Calculation and pipetting example for 10 reactions of the Master-Mix (ICD as extraction and PCR inhibition control)

Kit code	Master-Mix components	Volume per reaction	10 reactions (10 % extra)
1	Reaction Mix	19.3 µl	212.3 µl
2	Taq-Polymerase	0.7 µl	7.7 µl
	Total	**20 µl**	**220 µl**

Mix the components of the Master-Mix gently and briefly spin down.

Tab. 4: Calculation and pipetting example for 10 reactions of the Master-Mix (ICD only as PCR inhibition control)

Kit code	Master-Mix components	Volume per reaction	10 reactions (10 % extra)
1	Reaction Mix	19.3 µl	212.3 µl
2	Taq-Polymerase	0.7 µl	7.7 µl
D	Internal Control DNA	1.0 µl	11 µl
	Total	**21.0 µl**	**231.0 µl**

Mix the components of the Master-Mix gently and briefly spin down.

9.2 Preparation of the PCR-Mix

Pipette 20 µl of the Master-Mix in each reaction vial (tube or plate).

Negative control: Add 5 µl No Template Control to the pre-pipetted Master-Mix.

Note: If the Internal Control DNA is used as extraction control for the sample preparation procedure and as PCR inhibition control, we recommend to add 1 µl of the Internal Control DNA to the PCR-Mix of the negative control.

Sample: Add 5 µl DNA extract to the pre-pipetted Master-Mix.

Positive control: Add 5 µl Positive Control to the pre-pipetted Master-Mix.

Note: If the Internal Control DNA is used as extraction control for the sample preparation procedure and as PCR inhibition control, we recommend to add 1 µl of the Internal Control DNA to the PCR-Mix of the positive control.

Cover tubes or plate. Spin down and place in the real-time PCR instrument. The PCR reaction should be started according to the PCR instrument Set-up (see Tab. 5, Tab. 6, Tab.7, Tab. 8).

9.3 PCR instrument set-up

9.3.1 DNA real-time PCR profile

Tab. 5: DNA real-time PCR profile for LightCycler® series und Rotor-Gene Q

Initial Denaturation	1 min, 95 °C
Cycles	45 Cycles
PCR Denaturation Annealing/Extension	10 sec, 95 °C 15 sec, 60 °C
Temperature Transition Rate / Ramp Rate	Maximum

Note: Annealing and Extension occur in the same step.

Tab. 6: DNA real-time PCR profile for Mx3005P, ABI7500 und CFX96™

Initial Denaturation	1 min, 95 °C
Cycles	45 Cycles
PCR Denaturation Annealing/Extension	15 sec, 95 °C 30 sec, 60 °C
Temperature Transition Rate / Ramp Rate	Maximum

Note: Annealing and Extension occur in the same step.

9.3.2 Universal real-time PCR profile

Note: The universal real-time PCR profile should only be used for DNA assays when combining RIDA®GENE DNA and RNA real-time PCR assays in one run.

Tab. 7: Universal real-time PCR profile for LightCycler® series

Reverse Transcription	10 min, 58 °C
Initial Denaturation	1 min, 95 °C
Cycles	45 Cycles
PCR Denaturation Annealing/Extension	10 sec, 95 °C 15 sec, 60 °C
Temperature Transition Rate / Ramp Rate	Maximum

Note: Annealing and Extension occur in the same step.

Tab. 8: Universal real-time PCR profile for Mx3005P, ABI7500, Rotor-Gene Q and CFX96™

Reverse Transcription	10 min, 58 °C
Initial Denaturation	1 min, 95 °C
Cycles	45 Cycles
PCR Denaturation Annealing/Extension	15 sec, 95 °C 30 sec, 60 °C
Temperature Transition Rate / Ramp Rate	Maximum

Note: Annealing and Extension occur in the same step.

9.4 Detection channel set-up

Tab. 9: Selection of appropriate detection channels

Real-time PCR instrument	Detection	Detection Channel	Note
Roche LightCycler® 480II	*Clostridium difficile*	465/510	**RIDA®GENE Color Compensation Kit IV (PG0004) is required**
	ICD	533/580	
	C. difficile Toxin A/B Gen	618/660	
ABI 7500	*Clostridium difficile*	FAM	**Check that passive reference option ROX is none**
	ICD	VIC	
	C. difficile Toxin A/B Gen	Cy5	
Agilent Techn. Mx3005P	*Clostridium difficile*	FAM	**Check that reference dye is none**
	ICD	HEX	
	C. difficile Toxin A/B Gen	Cy5	
Qiagen Rotor-Gene Q	*Clostridium difficile*	Green	**The gain settings have to be set to 5, according to the default settings**
	ICD	Yellow	
	C. difficile Toxin A/B Gen	Red	
Bio-Rad CFX96™	*Clostridium difficile*	FAM	
	ICD	VIC	
	C. difficile Toxin A/B Gen	Cy5	

10. Quality Control

The analysis of the samples is done by the software of the used real-time PCR instrument according to the manufacturer` s instructions. Positive control and negative control have to show correct results (see Table 10, Fig. 1, Fig. 2) in order to determine a valid run.

The Positive Control for *Clostridium difficile* und *Clostridium difficile* toxin genes A/B has a concentration of 10^3 copies/µl. In each PCR run it is used in a total amount of 5 x 10^3 copies, respectively.

Tab. 10: For a valid run, the following conditions must be met:

Sample	Assay result	ICD Ct	Target Ct
Positive control	Positive	NA *[1]	See Quality Assurance Certificate
Negative control	Negative	Ct > 20	0

*[1] *No Ct value is required for the ICD to make a positive call for the positive control.*

If the positive control is not positive within the specified Ct range but the negative control is valid, prepare all new reactions including the controls.

If the negative control is not negative but the positive control is valid, prepare all new reactions including the controls.

If the required criteria are not met, following items have to be checked before repeating the test:

- Expiry of the used reagents
- Functionality of the used instrumentation
- Correct performance of the test procedure

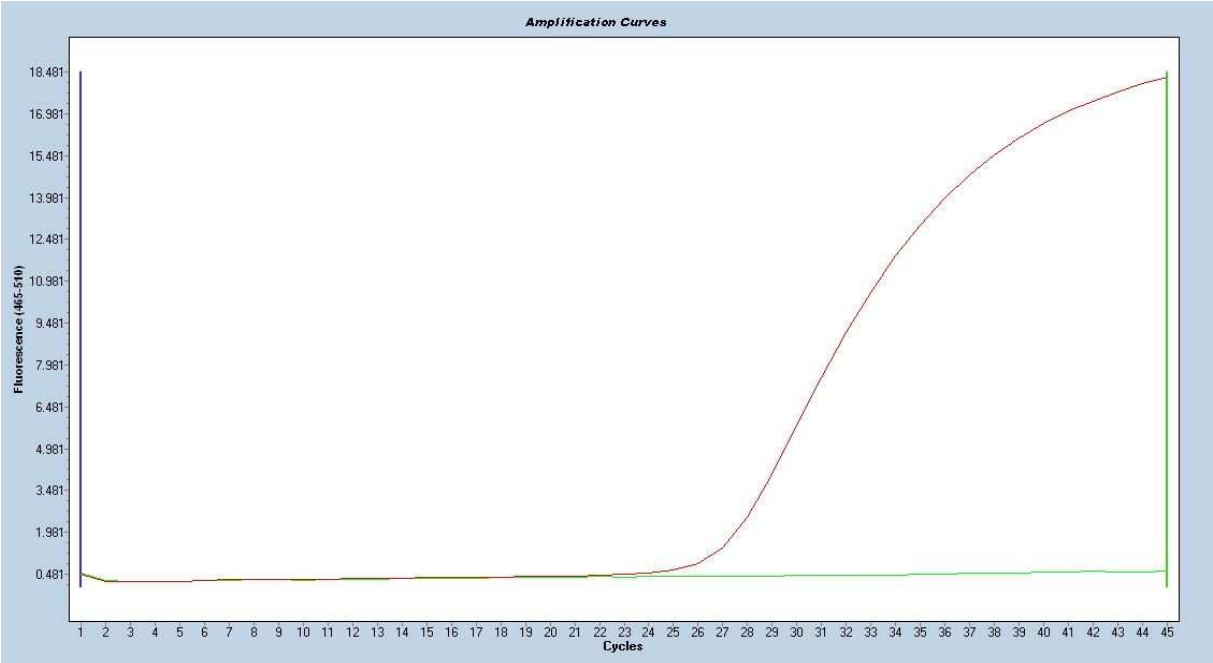

Fig. 1: Correct run of the positive and negative control (*Clostridium difficile*) on the LightCycler® 480II

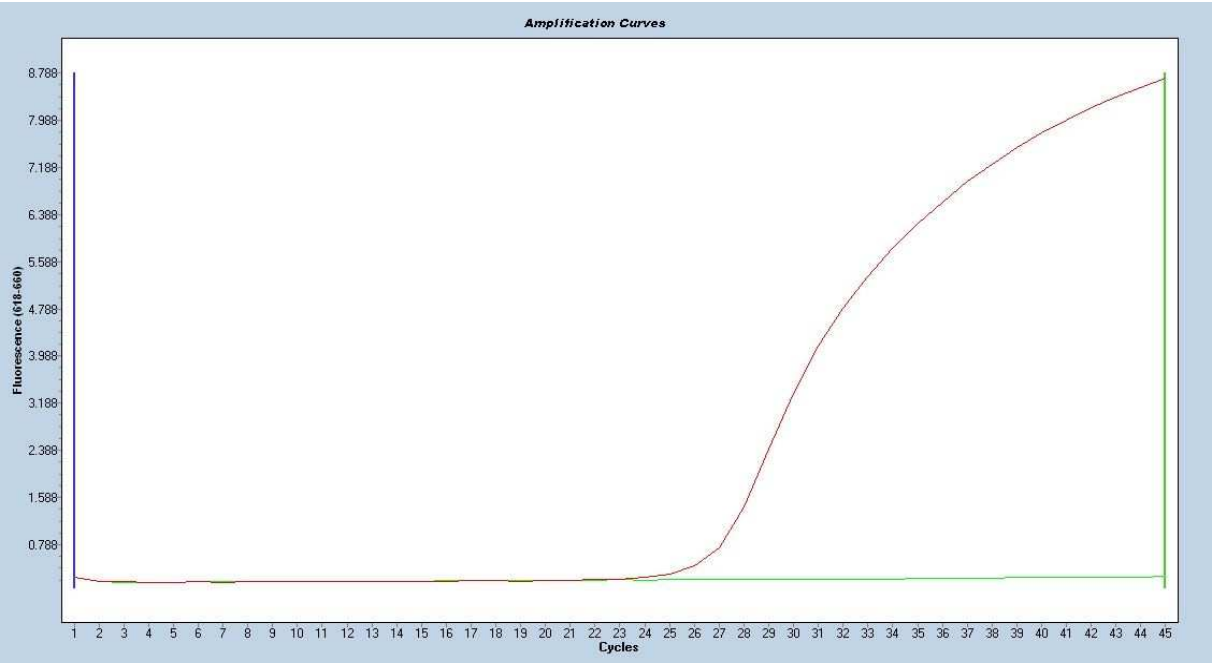

Fig. 2: Correct run of the positive and negative control (*C. difficile* toxin genes A/B) on the LightCycler® 480II

11. Result interpretation

The result interpretation is done according to Table 11.

Tab. 11: Sample interpretation

Target genes			
C. difficile	**Toxin genes A/B**	**ICD**	**Result**
positive	**positive**	**positive/negative**	**Toxigenic *C. difficile* strain detected**
positive	negative	**positive/negative**	**Non-toxigenic *C. difficile strain detected***
negative	**positive**	**positive/negative**	**Invalid**
negative	negative	**positive**	**Target genes not detected**
negative	negative	negative	**Invalid**

A toxigenic or non-toxigenic *C. difficile* strain is detected, if the sample DNA and the Internal Control DNA show an amplification signal in the detection system.

A toxigenic or non-toxigenic *C. difficile* strain is also detected, if the sample DNA shows an amplification signal but none for the Internal Control DNA in the detection system. The detection of the internal amplification control is not necessary because high concentrations of the amplicon can cause a weak or absent signal of the Internal Control DNA.

A toxigenic or non-toxigenic *C. difficile* strain is not detected, if the sample DNA shows no amplification signal, but an amplification signal for the Internal Control DNA in the detection system. An inhibition of the PCR reaction can be excluded by the detection of the Internal Control DNA.

A sample is invalid, if the sample DNA and Internal Control DNA show no amplification signal in the detection system. The sample contains a PCR inhibitor or a failure occurred in the extraction procedure. The extracted sample needs to be further diluted with PCR water (1:10) and re-amplified, or the isolation and purification of the sample has to be improved.

12. Limitations of the method

1. The result of molecular analysis should not lead to the diagnosis, but always be considered in the context of medical history and symptoms of the patient.
2. This assay is only suitable for stool and culture samples.
3. Inappropriate specimen collection, transport, storage and processing or a pathogen load in the specimen below the analytical sensitivity can result in false negative results.
4. The presence of PCR inhibitors may cause invalid results.
5. Mutations or polymorphisms in primer or probe binding regions may affect detection of new variants resulting in a false negative result with the RIDA®GENE Clostridium difficile assay.
6. As with all PCR based *in vitro* diagnostic tests, extremely low levels of target below the limit of detection (LoD) may be detected, but results may not be reproducible.
7. A positive test result does not necessarily indicate the presence of viable organisms. However, a positive result is indicative for the presence of target genes (16s-rDNA, tcdA/tcdB).

13. Performance characteristics

13.1 Analytical sensitivity

The RIDA®GENE Clostridium difficile multiplex real-time PCR has a detection limit of ≥ 10 DNA copies per reaction for *Clostridium difficile* and *C. difficile* toxin genes (see Fig. 3, Fig. 4).

The following figures 3 and 4 show dilution series of *Clostridium difficile* and *C. difficile* toxin genes (each 10^5 - 10^1 DNA copies per µl) on the LightCycler® 480II.

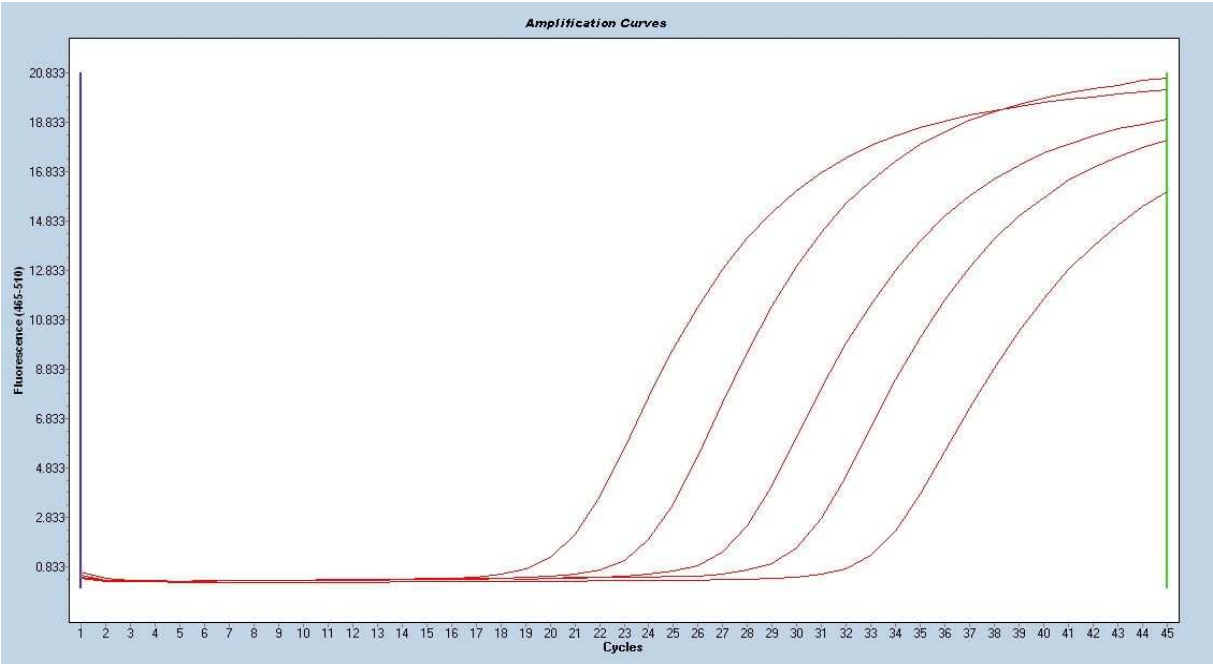

Fig. 3: Dilution series *Clostridium difficile* (10^5 - 10^1 DNA Copies / µl) on the LightCycler® 480II

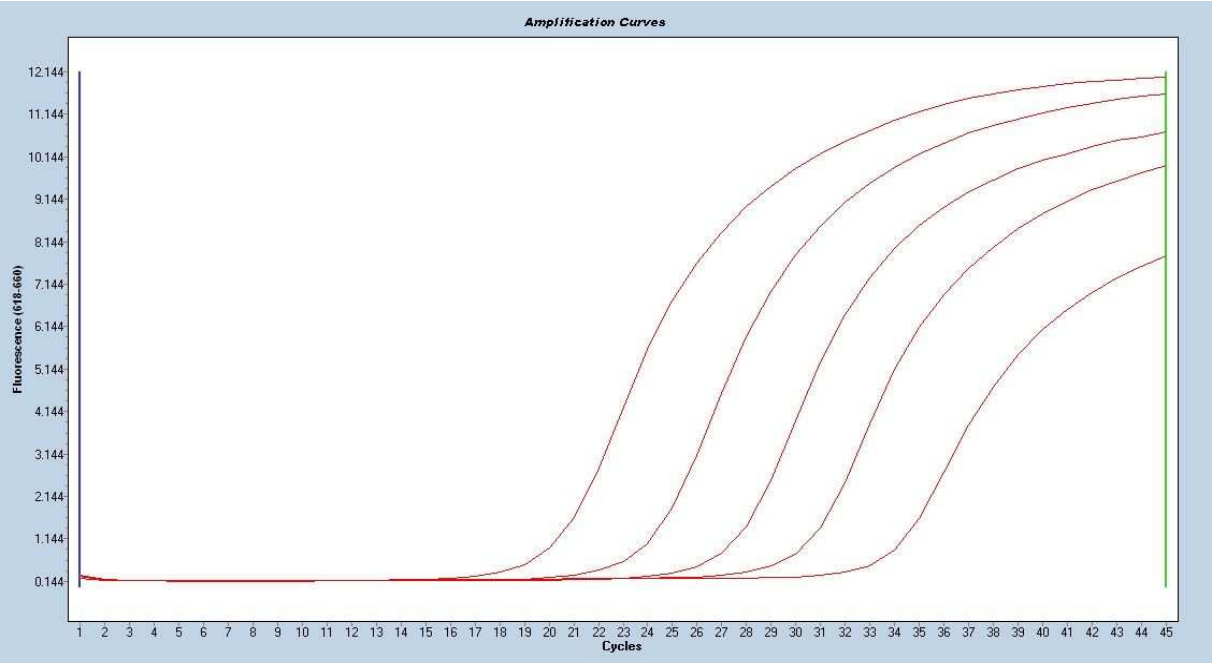

Fig. 4: Dilution series *C. difficile* toxin genes A/B (10^5 - 10^1 DNA Copies / µl) on the LightCycler® 480II

The detection limit of the whole procedure depends on the sample matrix, DNA extraction and DNA concentration.

13.2 Analytical specificity

The RIDA®GENE Clostridium difficile multiplex real-time PCR assay is specific for *Clostridium difficile* (16s-rDNA) and *C. difficile* toxin genes A/ B. No cross-reaction could be detected for the following species (see Tab. 12):

Tab. 12: Cross-reactivity testing

Adenovirus 1, human, strain Adenoid 71	-	*Campylobacter lari* subsp. *lari*	-	*Entamoeba histolytica*	-	*Proteus vulgaris*	-
Adenovirus 7, human, strain Gomen	-	*Campylobacter upsaliensis*	-	*E. coli* (O6)	-	*Pseudomonas aeruginosa*	-
Adenovirus 40, human, strain Dugan	-	*Candida albicans*	-	*E. coli* (O26:H-)	-	Rotavirus	-
Adenovirus 41, human, strain Tak	-	*Citrobacter freundii*	-	*E. coli* (O157:H7)	-	*Salmonella enteritidis*	-
Aeromonas hydrophila	-	*Clostridium bifermentans*	-	*Enterobacter cloacae*	-	*Salmonella typhimurium*	-
Arcobacter butzleri	-	*Clostridium novyi*	-	*Enterococcus faecalis*	-	*Serratia liquefaciens*	-
Astrovirus	-	*Clostridium perfringens*	-	*Giardia intestinalis* Portland 1	-	*Shigella flexneri*	-
Bacillus cereus	-	*Clostridium septicum*	-	*Giardia intestinalis* WB Clone C6	-	*Staphylococcus aureus*	-
Bacteroides fragilis	-	*Clostridium sordellii*	-	*Giardia lamblia*	-	*Staphylococcus epidermidis*	-
Campylobacter coli	-	*Clostridium sporogenes*	-	*Klebsiella oxytoca*	-	*Vibrio parahaemolyticus*	-
Campylobacter fetus subsp. *fetus*	-	*Cryptosporidium muris*	-	Norovirus GG I	-	*Yersinia enterocolitica*	-
Campylobacter jejuni	-	*Cryptosporidium parvum*	-	Norovirus GG II	-		

13.3 Analytical reactivity

The reactivity of the RIDA®GENE Parasitic Stool Panel II multiplex real-time PCR assay was tested with *Clostridium difficile* (see Tab. 13). All *Clostridium difficile* strains tested were detected by the RIDA®GENE Parasitic Stool Panel II multiplex real-time PCR assay or by sequence alignment.

Tab. 13: Analytical reactivity testing

Clostridium difficile					
C. difficile Ribotyp 001	+	*C. difficile* Ribotyp 027	+	*C. difficile* Ribotyp 078	+
C. difficile Ribotyp 002	+	*C. difficile* Ribotyp 046	+	*C. difficile* Ribotyp 0126	+
C. difficile Ribotyp 017	+	*C. difficile* Ribotyp 056	+	*C. difficile* Ribotyp 0131	+
C. difficile Ribotyp 020	+	*C. difficile* Ribotyp 075	+	*C. difficile* Ribotyp FK012	+
C. difficile Ribotyp 023	+				
C. difficile toxinotype 0	+	*C. difficile* toxinotype X	+	*C. difficile* toxinotype XXI	+
C. difficile toxinotype I	+	*C. difficile* toxinotype XIa	+	*C. difficile* toxinotype XXII	+
C. difficile toxinotype II	+	*C. difficile* toxinotype XIb	+	*C. difficile* toxinotype XXIV	+
C. difficile toxinotype IIIa	+	*C. difficile* toxinotype XIc	+	*C. difficile* toxinotype XXV	+
C. difficile toxinotype IIIb	+	*C. difficile* toxinotype XId	+	*C. difficile* toxinotype XXVI	+
C. difficile toxinotype IIIc	+	*C. difficile* toxinotype XII	+	*C. difficile* toxinotype XXVII	+
C. difficile toxinotype IV	+	*C. difficile* toxinotype XIII	+	*C. difficile* toxinotype XXVIII	+
C. difficile toxinotype V	+	*C. difficile* toxinotype XIVa	+	*C. difficile* toxinotype XXIX	+
C. difficile toxinotype VI	+	*C. difficile* toxinotype XIVb	+	*C. difficile* toxinotype XXX	+
C. difficile toxinotype VII	+	*C. difficile* toxinotype XVI	+	*C. difficile* toxinotype XXXI	+
C. difficile toxinotype VIII	+	*C. difficile* toxinotype XVII	+	*C. difficile* toxinotype XXXII	+
C. difficile toxinotype IXa	+	*C. difficile* toxinotype XVIII	+	*C. difficile* toxinotype XXXIII	+
C. difficile toxinotype IXb	+	*C. difficile* toxinotype XIX	+	*C. difficile* toxinotype XXXIV	+
C. difficile toxinotype IXc	+	*C. difficile* toxinotype XX	+		

14. Version history

Version number	Chapter and designation
2013-04-17	Release version
2018-05-11	General revision
2018-05-11	4. Reagents provided 6. Additional necessary reagents and necessary equipment 9. Test procedure 10. Quality control 13. Performance characteristics 14. Version history 15. Explanation of symbols

15. Explanation of symbols

General symbols

IVD	For *in vitro* diagnostic use
	Consult instructions for use
LOT	Lot number
	Expiry
	Store at
REF	Article number
Σ	Number of tests
	Date of manufacture
	Manufacturer

Testspecific symbols

Not applicable

16. Literature

1. Hall IC and O'Toole E. Intestinal flora in new-born infants: with a description of a new pathogenic anaerobe, Bacillus difficilis. *Am J Dis Child* 1935, 49: 390–402.
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3. Bartlett JG and Gerding DN. Clinical Recognition and Diagnosis of Clostridium difficile Infection. CID 2008, 46: S12-18.
4. Bartlett JG. Narrative Review: The new Epidemic of Clostridium difficile-Associated Enteric Disease. *Ann Intern Med* 2006; 145:758-764.