RIDA®GENE CD Toxin A/B

real-time PCR

Art. No.: PG0825

100 reactions

For *in vitro* diagnostic use.

-20 °C

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1. Intended use

For *in vitro* diagnostic use. RIDA®GENE CD Toxin A/B is a real-time PCR for the direct, qualitative detection of *Clostridium difficile* toxin A (tcdA) and toxin B (tcdB) genes in human stool samples. The RIDA®GENE CD Toxin A/B real-time PCR is intended for use as an aid in diagnosis of *Clostridium difficile* associated diarrhea (CDAD).

2. Explanation

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. 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. [4]
Real-time PCR permits a rapid, highly sensitive and specific detection of Clostridium difficilce 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

RIDA®GENE CD Toxin A/B real-time PCR is for the direct, qualitative detection of *Clostridium difficile* toxin A (tcdA) and toxin B (tcdB) genes in human stool samples.
After DNA-Isolation, amplification of the gene fragments specific for toxin A and B of *Clostridium difficile* (if present) occurs. The amplified DNA 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 instrument. The fluorescence signal increases with the amount of formed amplicons. The RIDA®GENE CD Toxin A/B assay contains an Internal Control DNA (ICD) as an internal control of sample preparation procedure and 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 1100 µl	yellow
2	Taq-Polymerase	1x 11 µl	red
D	Internal Control DNA	2x 1800 µl	orange
N	PCR Water	1x 500 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 5 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 equipment and materials required

- DNA-Extraction kit for stool samples (e.g. RTP® Pathogen Kit (STRATEC Molecular)) or
  DNA-Extraction system for stool samples (e.g. Maxwell® 16 (Promega), NucliSENS easy®MAG™ (bioMérieux))
- Real-time PCR Instrument:

Roche:	LightCycler® 2.0, LightCycler® 480II
Cepheid:	SmartCycler®
Applied Biosystems:	ABI 7500
Abbott:	m2000rt
Stratagene:	Mx3000P, Mx3005P
QIAGEN:	Rotor-Gene Q

- RIDA®GENE Color Compensation II (PG0002) for run the LightCycler® 2.0
- 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

7. Precautions for users

- For *in vitro* diagnostic use only.
- Extraction, PCR preparation and the PCR run should be separated in different rooms to avoid cross-contaminations.
- This test must only be performed by laboratory personnel trained in molecular biology methods.
- Strictly follow the working instructions.
- When handling samples, wear disposable gloves. After finishing the test, wash your hands.
- Do not smoke, eat or drink in areas where samples or test reagents are being used.
- 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.

8. Test procedure

8.1 Sample Preparation

8.1.1 DNA-Isolation from Stool Samples

Use for DNA-isolation of human stool samples a commercially available DNA isolation kit (e.g. RTP® Pathogen Kit (STRATEC Molecular)) or DNA extraction system (e.g. Maxwell® 16 (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 3,000 rpm for 30 sec. Use from the supernatant the appropriate volume according to the manufacturer's instruction.

The RIDA®GENE CD Toxin A/B assay contains an Internal Control DNA (ICD), which can either be used as PCR inhibition control or as extraction control for the sample preparation procedure and as a PCR inhibition control.

If the ICD is used only as a PCR inhibition control, 1µl of the ICD should be added to the Master-Mix (see Tab.3).

If the ICD is used as an extraction control for the sample preparation procedure **and** as PCR inhibition control, 20 µl of the ICD has to be added during extraction procedure. The ICD should always be added to the specimen-lysis buffer mixture and must **not** be added directly to the specimen.

8.2 Master-Mix preparation

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

We recommend to calculate an additional volume of 10 % to compensate imprecise pipetting (see Tab.2, Tab.3). Thaw, mix gently and centrifuge briefly the Reaction Mix, the Taq-Polymerase, the Positive Control, the PCR Water and the ICD before using. Keep reagents appropriately cold during working step (2 - 8 °C).

Tab.2: 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	19.9 µl	218.9 µl
2	Taq-Polymerase	0.1 µl	1.1 µl
	Total	**20.0 µl**	**220 µl**

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

Tab.3: 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	19.9 µl	218.9 µl
2	Taq-Polymerase	0.1 µl	1.1 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.

8.3 Preparation of the PCR-Mix

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

Negative control: Add 5 µl PCR Water as negative control to the pre-pipetted Master-Mix.

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

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 ICD is used as extraction control for the sample preparation procedure* ***and*** *as PCR inhibition control, we recommend to add 1 µl of the ICD to the positive control PCR Mix.*

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.4, Tab. 5).

8.4 PCR Instrument Set-up

Tab.4: Real-time PCR profile for LightCycler® 2.0, LightCycler® 480II, SmartCycler® and Rotor-Gene Q

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

Note: *Annealing and Extension occur in the same step.*

Tab.5: Real-time PCR profile for Mx3000P, Mx3005P, ABI 7500 and m2000rt

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

Note: *Annealing and Extension occur in the same step.*

8.5 Detection Channel Set-up

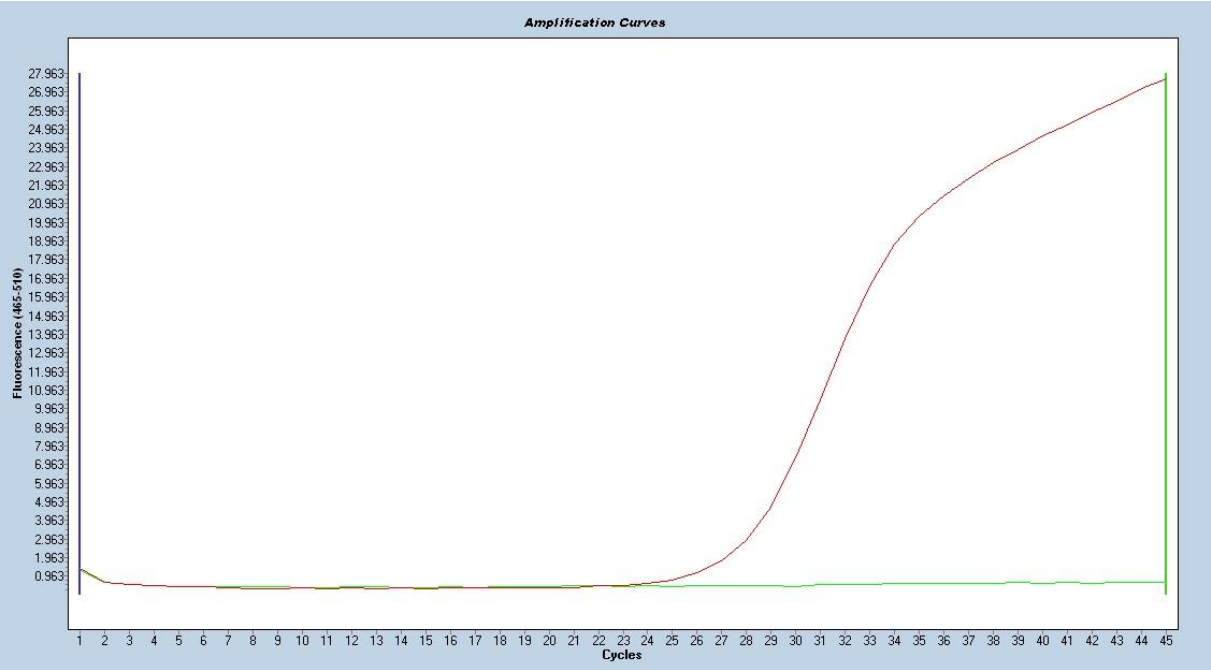
Tab.6: Selection of appropriate detection channels

Real-time PCR instrument	Detection	Detection Channel	Dark-Quencher	Note
Roche LightCycler® 2.0	*Clostridium difficile* Toxin A/B	530	+	**RIDA®GENE Color Compensation Kit II (PG0002) is required**
	ICD	560	+	
Roche LightCycler® 480II	*Clostridium difficile* Toxin A/B	465/510	+	**Color Compensation is not required**
	ICD	533/580	+	
Cepheid SmartCycler®	*Clostridium difficile* Toxin A/B	Channel 1	+	**-**
	ICD	Channel 2	+	
ABI 7500	*Clostridium difficile* Toxin A/B	FAM	none	**Check that passive reference option ROX is none**
	ICD	VIC	none	
Abbott m2000rt	*Clostridium difficile* Toxin A/B	FAM	none	**-**
	ICD	VIC	none	
Stratagene Mx3000P/ Mx3005P	*Clostridium difficile* Toxin A/B	FAM	+	**Check that reference dye is none**
	ICD	HEX	+	
Qiagen Rotor-Gene Q	*Clostridium difficile* Toxin A/B	Green	+	**-**
	ICD	Yellow	+	

9. Result interpretation

The analysis of the samples is done by the software of the used real-time PCR instrument according to the manufacturer` s instructions. Positive and negative controls have to show correct results (see Fig. 1). The positive control 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.

Fig.1: Correct run of the positive and negative control (*C. difficile* toxin A/B genes) on the LightCycler® 480II



The result interpretation is done according to Table 7.

Tab.7: Sample interpretation

Sample	ICD	Result
-	+	**Sample negative**
+	+	**Sample positive**
+	-	**Sample positive, competition**
-	-	**PCR inhibition**

A sample is evaluated negative, if the sample shows no amplification signal in the detection system, but the Internal Control DNA (ICD) is positive. An inhibition of the PCR reaction or a failure in the extraction procedure can be excluded by the detection of the Internal Control DNA (ICD).

A sample is evaluated positive, if both, the sample and the Internal Control DNA, (ICD) show an amplification signal in the detection system.

A sample is evaluated positive, if the sample shows an amplification signal in the detection system, but the Internal Control DNA (ICD) is negative. 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 amplification control.

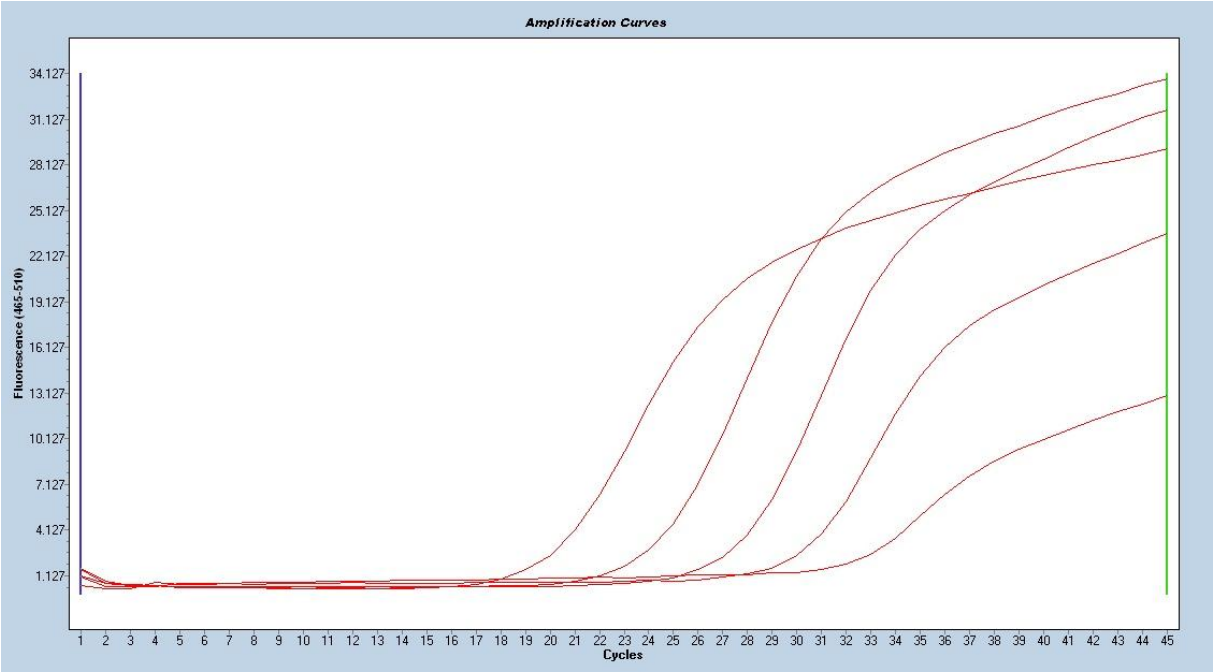
A sample is evaluated invalid, if both, the sample and the Internal Control DNA (ICD) show no amplification signal in the detection system. The sample contained a PCR inhibitor or a failure occurred in the extraction procedure. The extracted sample needs to be further diluted with PCR water (1:10) and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

10. Test characteristics

10.1 Analytical sensitivity

The RIDA®GENE CD Toxin A/B real-time PCR has detection limit of ≤ 5 DNA copies per reaction (see Fig. 2).

Fig.2: Dilution series *C difficile* toxin A/B genes (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.

10.2 Analytical specificity

The RIDA®GENE CD Toxin A/B real-time PCR is specific for *Clostridium difficile* in human stool samples. No cross-reaction could be detected for the following species (see Tab.8):

Tab. 8: Cross-reactivity testing

Arcobacter butzleri	-	*Clostridium sordellii*	-	*Salmonella enteritidis*	-
Aeromonas hydrophila	-	Enteropathogenic *E.coli*	-	*Salmonella typhimurium*	-
Bacillus cereus	-	Enterotoxigenic *E.coli*	-	*Serratia liquefaciens*	-
Bacteroides fragilis	-	Shiga toxin-producing *E.coli*	-	*Shigella flexneri*	-
Campylobacter coli	-	*Enterobacter cloacae*	-	*Staphylococcus aureus*	-
Campylobacter jejuni	-	*Enterococcus faecalis*	-	*Staphylococcus epidermidis*	-
Candida albicans	-	*Klebsiella oxytoca*	-	*Vibrio parahaemolyticus*	-
Citrobacter freundii	-	*Proteus vulgaris*	-	*Yersinia enterocolitica*	-
Clostridium perfringens	-	*Pseudomonas aeruginosa*	-		

11. 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 validated for stool 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 CD Toxin A/B 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 the *C. difficile* toxin A/B genes (tcdA/tcdB).

12. 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.
2. Bartlett JG, et al. Antibiotica-associated pseudomembranous colitis due to a toxin-producing clostridia. *N Engl J Med*, 1978; 298:531-543.
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.