Instruction for Use

diarellaC.difficile

real time PCR Kit

For *in vitro* detection of toxin A (tcdA) and toxin B (tcdB) specific DNA of Clostridium difficile extracted from biological specimens.

REF **G01147-96**

96

- 18°C

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Index

1 Intended Purpose

The Kit is designed for the qualitative detection of the nucleic acid of Clostridium difficile toxin A and Clostridium difficile toxin B in eluates from biological specimens. The assay is an in vitro diagnostic medical device and intended to be used by professional users in a laboratory environment. It can be performed manually or using an automated platform. The assay serves as an aid in the diagnosis, screening and monitoring of Clostridium difficile.

2 Pathogen Information

Clostridium difficile is a spore-forming, anaerobic gram-positive bacterium from the genus Clostridium. The species encompasses a diverse population structure with hundreds of different strain types. The bacteria are transmitted via the faecal-oral route, often during hospitalisation, this is due to improper isolation of infected patients and poor hygienic routines. But not all infected patients show symptoms. Clostridium difficile cannot be found in the normal, healthy gastrointestinal flora because other bacteria interfere with its multiplication [1].

While some strains of the bacteria are non-toxigenic (NTCD) other strains can produce either one or two toxins, Toxin A (tcdA) and B (tcdB). The toxins produced disrupt the adherence of the mucosal cells (tcdA) and induce apoptosis (tcdB), which can lead to various pathologies ranging from mild diarrhoea to life-threatening inflammatory complications such as pseudomembranous colitis or toxic megacolon. In 1.5 % of all hospitalised cases of Clostridium difficile diarrhoea, the infection is fatal, with the risk being highest in elderly patients.

3 Principle of the Test

The diarellaC.difficile real time PCR contains specific primers and dual-labeled probes for the amplification of the DNA of the Clostridium difficile specific toxin A (tcdA) and toxin B (tcdB) extracted from biological specimens.

The presence of nucleic acid is detected by an increase in fluorescence due to hydrolysis of the probes during amplification. The fluorescence of the tcdA specific probes is measured in the FAM channel. The fluorescence of the tcdB specific probes is measured in the Cy5 channel.

Furthermore, diarellaC.difficile real time PCR contains a Control DNA (Internal Process Control, IPC), which is added during DNA extraction and detected in the same reaction by a HEX-labeled probe.

The Control DNA allows the detection of PCR inhibition and acts as control, that the nucleic acid was isolated from the biological specimen.

4 Package Contents

The reagents supplied are sufficient for 96 reactions.

Table 1: Components of the diarellaC.difficile real time PCR Kit

Label	Lid Colour	Content 96
Reaction Mix	yellow	1 x 1344 µl
Positive Control	red	1 x 150 µl
Negative Control	green	1 x 150 µl
Control DNA	colourless	1 x 480 µl

5 Equipment and Reagents to be Supplied by User

- DNA isolation kit (e.g. NukEx Mag RNA/DNA, gerbion Cat. No. G05012)
- PCR grade Water
- Sterile microtubes
- Pipets (adjustable volume)
- Sterile pipet tips with filter
- Table centrifuge
- Vortexer
- Real time PCR instrument
- Optical PCR reaction tubes with lid or optical PCR reaction plate with optical foil
- Optional: Liquid handling system for automation

6 Transport, Storage and Stability

The diarellaC.difficile real time PCR Kit is shipped on dry ice. All components must be stored at maximum -18°C in the dark immediately after receipt. Do not use reagents after the date of expiry printed on the package. Up to 20 freeze and thaw cycles are possible.
Protect kit components from direct sunlight during the complete test run.

7 Warnings and Precautions

Read the Instructions for Use carefully before using the product.
Before first use check the product and its components for:

- Use of this product is limited to personnel specially instructed and trained in the techniques of real time PCR procedures.
- Specimens should always be treated as infectious and/or biohazardous in accordance with safe laboratory procedures.
- Avoid microbial and nuclease (DNase/RNase) contamination of the eluates and the components of the kit.
- Always use DNase/RNase-free disposable pipette tips with aerosol barriers.
- Always wear protective disposable powder-free gloves when handling kit components.
- Use separated and segregated working areas for (1) sample preparation, (2) reaction setup and (3) amplification/detection activities. The workflow in the laboratory should proceed in unidirectional manner. Always wear disposable gloves in each area and change them before entering a different area.
- Dedicate supplies and equipment to the separate working areas and do not move them from one area to another.
- Store positive and/or potentially positive material separated from all other components of the kit.
- Do not open the reaction tubes/plates post amplification to avoid contamination with amplicons.
- Additional controls may be tested according to guidelines or requirements of local, state and/or federal regulations or accrediting organizations.
- Do not autoclave reaction tubes after the PCR, since this will not degrade the amplified nucleic acid and will bear the risk to contaminate the laboratory area.
- Discard sample and assay waste according to your local safety regulations.
- Do not mix components from different lots.

8 Sample Material

Starting material for diarellaC.difficile real time PCR is DNA isolated from biological specimens. By the nature of the pathogens, sample material like stool is commonly used.

9 Sample Preparation

Commercial kits for DNA isolation such as the following are recommended:

- NukEx Mag RNA/DNA, gerbion Cat. No. G05012

Please follow the instructions for use of the respective extraction kit.

Important:

In addition to the samples always run a ‚water control' in your extraction. Treat this water control analogous to a sample.

Comparing the amplification of the Control DNA in the samples to the amplification of the internal control in the water control will give insights on possible inhibitions of the real time PCR. Furthermore, possible contaminations during DNA extraction will be detectable.

Please note the chapter ‚Control DNA'.

If the real time PCR is not performed immediately, store extracted DNA according to the instructions given by the manufacturer of the respective nucleic acid extraction kit.

10 Control DNA

A Control DNA is supplied as extraction control. This allows the user to control the DNA isolation procedure and to check for possible real time PCR inhibition.

Add 5 µl Control DNA per extraction (5 µl x (N+1)). Mix well. Perform the DNA isolation according to the manufacturer's instructions.

The Control DNA must be added to the Lysis Buffer of the extraction kit.

11 Real time PCR

11.1 Important Points Before Starting:

- Please pay attention to the chapter 7 ‚Warnings and Precautions'.
- Before setting up the real time PCR familiarize yourself with the real time PCR instrument and read the user manual supplied with the instrument.
- The programming of the thermal profile should take place before the PCR set up.
- In every PCR run one Positive Control and one Negative Control should be included.

- Before each use, all reagents should be thawed completely at room temperature, thoroughly mixed, and centrifuged very briefly.

11.2 Procedure

The Master Mix contains all the components needed for PCR except the sample. Prepare a volume of Master Mix for at least one sample more than required, in order to compensate for pipetting inaccuracy.

Table 2: Preparation of the Master Mix

Volume per Reaction	Volume Master Mix
14.0 µl Reaction Mix	14.0 µl x (N+1)

Real time PCR set-up

- Place the number of optical PCR reaction tubes needed into the respective tray of the real time PCR instrument / take an optical PCR reaction plate.
- Pipet **14 µl** of the Master Mix into each optical PCR reaction tube / the optical PCR reaction plate.
- Add **6 µl** of the eluates from the DNA isolation (including the eluate of the water control), the Positive Control and the Negative Control to the corresponding optical PCR reaction tube / the optical PCR reaction plate (Table 3).
- Close the optical PCR reaction tubes / the optical PCR reaction plate immediately after filling in order to reduce the risk of contamination.

Table 3: Preparation of the real time PCR

Component	Volume
Master Mix	14.0 µl
Sample	6.0 µl
Total Volume	20.0 µl

11.3 Instrument Settings

For the real time PCR use the thermal profiles shown in Table 4.

Table 4: real time PCR thermal profile

Description	Time	Temperature	Number of Cycles
Initial Denaturation	5 min	95°C	1
Amplification of DNA			
Denaturation	10 sec	95°C	45
Annealing and Extension	40 sec	60°C	
	Acquisition at the end of this step		

If in the same run samples should be tested for pathogens with RNA genome, use the thermal profile shown in Table 5.

Table 5: real time RT-PCR thermal profile

Description	Time	Temperature	Number of Cycles
Reverse Transcription	10 min	45 °C	1
Initial Denaturation	5 min	95°C	1
Amplification of DNA			
Denaturation	10 sec	95°C	45
Annealing and Extension	40 sec	60°C	
	Acquisition at the end of this step		

Dependent on the real time instrument used, further instrument settings must be adjusted according to Table 6.

Table 6: Overview of the instrument settings required for the diarellaC.difficile real time PCR.

Real time PCR Instrument	Parameter	Detection Channel	Notes		
LightCycler 480II			Color Compensation Kit Multiplex 1 (G070MP1-CC) required		
			Melt Factor	Quant Factor	Max Integration Time (sec)
	C. difficile toxin A	465-510	1	10	1
	Control DNA (IPC)	533-580	1	10	2
	C. difficile toxin B	618-660	1	10	3
Stratagene Mx3000P / Mx3005P	C. difficile toxin A	FAM	Gain 8	Reference Dye: None	
	Control DNA (IPC)	HEX	Gain 1		
	C. difficile toxin B	Cy5	Gain 4		
QuantStudio 5 CFX96 CFX Opus 96 Aria Mx qTower³ G	C. difficile toxin A	FAM	Option Reference Dye ROX: NO		
	Control DNA (IPC)	HEX			
	C. difficile toxin B	Cy5			
Mic qPCR Cycler	C. difficile toxin A	Green	Gain 8		
	Control DNA (IPC)	Yellow	Gain 10		
	C. difficile toxin B	Red	Gain 10		

12 Data Analysis

Following results can occur:

Signal/C_T Values			**Interpretation**
FAM Channel C. difficile **Toxin A**	**Cy5 Channel** C. difficile **Toxin B**	**HEX Channel** Control DNA (IPC)	
positive	negative	positive or negative[1]	**Positive result,** the sample contains Clostridium difficile toxin A DNA.
negative	**positive**	positive or negative[1]	**Positive result,** the sample contains Clostridium difficile toxin B DNA.
positive	**positive**	positive or negative[1]	**Positive result,** the sample contains Clostridium difficile toxin A and toxin B DNA.
negative	negative	≤ 34 [2]	**Negative result**, the sample contains no Clostridium difficile toxin A or toxin B DNA.
negative	negative	negative or > 34 [2]	**No diagnostic statement can be made.** The real time PCR is either inhibited or errors occurred while DNA extraction.

[1] A strong positive signal in the FAM or the Cy5 can inhibit the IPC. In such cases the result for the Control DNA can be neglected.

[2] In case of high C_T values, the IPC should be compared to the water extraction control as described in the chapter 'Assay validation'.

13 Assay Validation

Negative Controls

The Negative Control must show no C_T in the FAM, Cy5 and HEX channel.

Positive Controls

The Positive Control must show a positive (i.e. exponential) amplification curve in the different channels FAM and Cy5. The Positive Control must fall below C_T 30.

Internal Controls

The following values for the amplification of the internal controls are valid using gerbion nucleic acid extraction kit NukEx Mag RNA/DNA. The Control DNA (IPC) must show a positive (i.e. exponential) amplification curve.

The IPC must fall below a C_T of 34. If the IPC is above C_T 34 this points to a purification problem or a strong positive sample that can inhibit the IPC. In the latter case, the assay is valid. It is recommended to perform the extraction of a water control in each run. The IPC in the water control must fall below a C_T of 34.

If other nucleic acid extraction kits are used, the customer must define own cutoffs. In this case the C_T value of the Control DNA (IPC) in an eluate from a sample should not be delayed for more than 4 C_T in comparison to an eluate from an extracted water control.

14 Limitations of the Method

- Strict compliance with the Instruction for Use is required for optimal results.
- Use of this product is limited to personnel specially instructed and trained in the techniques of real time PCR and in vitro diagnostic procedures.
- Good laboratory practice is essential for proper performance of this assay.
- All reagents should be closely monitored for impurity and contamination. Any suspicious reagents should be discarded.
- This assay must not be used on a biological specimen directly. Appropriate nucleic acid extraction methods have to be conducted prior to using this assay.
- The presence of PCR inhibitors may cause false negative or invalid results.
- As with any diagnostic test, results of the diarellaC.difficile real time PCR Kit need to be interpreted in consideration of all clinical and laboratory findings.

15 Troubleshooting

The following troubleshooting guide is included to help you with possible problems that may arise when performing a real time PCR. If you have further questions, please do not hesitate to contact our scientists on info@gerbion.com.

No fluorescence signal in the FAM and/or Cy5 channel of the Positive Control	
The selected channel for analysis does not comply with the protocol	Select the FAM channel for analysis of the C. difficile toxin A specific amplification, the Cy5 channel for the C. difficile toxin B specific amplification and the HEX channel for the amplification of the Control DNA (IPC).
Incorrect configuration of the real time PCR	Check your work steps and compare with chapter 'Procedure'.
The programming of the thermal profile is incorrect	Compare the thermal profile with the protocol described in chapter ´Instrument Settings´
Incorrect storage conditions for one or more kit components or kit expired	Check the storage conditions and the date of expiry printed on the kit label. If necessary, use a new kit and make sure kit components are stored as described in chapter 'Transport, Storage and Stability'.
Weak or no signal of the Control DNA (IPC) and simultaneous absence of a signal in the specific FAM and/or Cy5 channel.	
real time PCR conditions do not comply with the protocol	Check the real time PCR conditions (chapter ´Real time PCR´).
real time PCR inhibited	Make sure that you use an appropriate isolation method (see chapter ´Sample Preparation´) and follow the manufacturer's instructions. Make sure that the ethanol-containing washing buffers have been completely removed.
sample material not sufficient	Make sure that enough sample material has been applied to the extraction. Use an appropriate isolation method (see chapter ´Sample Preparation´) and follow the manufacturer's instructions.
DNA loss during isolation process	In case the Control DNA was added before extraction, the lack of an amplification signal can indicate that the DNA isolation was not successful. Make sure that you use an appropriate isolation method (commercial kits are recommended) and stick to the manufacturer's protocol.
Incorrect storage conditions for one or more components or kit expired	Check the storage conditions and the date of expiry printed on the kit label. If necessary, use a new kit and make sure kit components are stored as described in chapter 'Transport, Storage and Stability'.

Detection of a fluorescence signal in the FAM and/or Cy5 channel of the Negative Control	
Contamination during preparation of the PCR	Repeat the real time PCR in replicates. If the result is negative in the repetition, the contamination occurred when the samples were pipetted into the optical PCR reaction tubes. Make sure to pipet the Positive Control last and close the optical PCR reaction tube immediately after adding the sample. If the same result occurs, one or more of the kit components might be contaminated. Make sure that work space and instruments are decontaminated regularly. Use a new kit and repeat the real time PCR.

16 Kit Performance

16.1 Analytical Sensitivity

For the FAM and Cy5 channels, the limits of detection (LoD) of diarellaC.difficile real time PCR Kit were determined using serial dilutions of the of synthetic DNA-fragments containing the specific gene target sequence. The determination of the LoD was done on a CFX96 Touch Instrument (Bio-Rad).

The LoD of diarellaC.difficile real time PCR Kit is $\leq$ 0.5 genome copies per µl for the FAM and $\leq$ 2.5 genome copies per µl for the Cy5 channel.

16.2 Analytical Specificity

The specificity of the diarellaC.difficile real time PCR was evaluated with different ring trial samples of known status and different other relevant viruses and bacteria found in biological samples and basing on in silico analyses.
All ring trial samples and other eluates with known status were detected correctly. Results are shown in Table 7 and Table 8.

Table 7: Ring trial samples tested for the validation of the sensitivity and specificity of the diarellaC.difficile real time PCR Kit.

sample	C. difficile toxin A	C. difficile toxin B
	FAM channel	Cy5 channel
QCMD 2020 Clostridium difficile DNA I		
CDDNA101S-01 C. difficile ribotype 027	**positive**	**positive**
CDDNA101S-02 Negative	negative	negative
CDDNA101S-03 C. difficile ribotype 002 *	**positive**	**positive**
CDDNA101S-04 C. difficile ribotype 002	**positive**	**positive**
CDDNA101S-05 C. difficile ribotype 002	**positive**	**positive**
CDDNA101S-06 C. difficile ribotype 027 *	**positive**	**positive**
CDDNA101S-07 C. difficile ribotype 027	**positive**	**positive**
CDDNA101S-08 C. difficile ribotype 078	**positive**	**positive**
CDDNA101S-09 C. difficile ribotype 078 *	**positive**	**positive**
CDDNA101S-10 C. difficile ribotype 078	**positive**	**positive**

* Educational sample, very low amount of target copies.

Table 8: Eluted DNA/RNA from bacterial and viral pathogens tested for the determination of the analytical specificity of diarellaC.difficile real time PCR Kit.

Eluates with known status	**diarellaC.difficile** toxin A **FAM channel**	**diarellaC.difficile** toxin B **Cy5 channel**
Astrovirus	negative	negative
Campylobacter coli	negative	negative
Campylobacter jejunii	negative	negative
Campylobacter lari	negative	negative
Enterococcus faecalis	negative	negative
Escherichia coli 0157	negative	negative
Norovirus GI	negative	negative
Norovirus GII	negative	negative
Rotavirus	negative	negative
Salmonella enterica	negative	negative
Sapovirus	negative	negative
Shigella flexneri	negative	negative
Yersinia enterocolitica	negative	negative
Listeria monocytogenes	negative	negative
Staphylococcus aureus	negative	negative
Enterovirus 71	negative	negative
Campylobacter jejuni	negative	negative
Campylobacter lari	negative	negative
Clostridium difficile 027	**positive**	**positive**
Salmonella enteritidis	negative	negative
Shigella flexneri	negative	negative
Yersinia enterocolitica	negative	negative
Giardia lamblia	negative	negative
Cryptosporidium parvum	negative	negative
Entamoeba histolytica	negative	negative
Plesiomonas shigelloides	negative	negative

16.3 Linear Range

The linear range of the diarellaC.difficile real time PCR Kit was evaluated by analysing logarithmic dilution series of quantified synthetic DNAs of the target sequences.

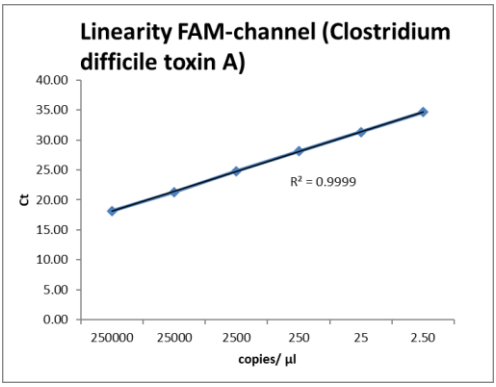

Figure 1: Determination of the linear range of the diarellaC.difficile real time PCR Kit for Clostridium difficile toxin A in the FAM channel:

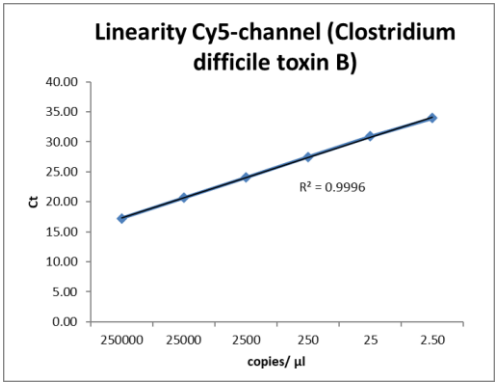

Figure 2: Determination of the linear range of the diarellaC.difficile real time PCR Kit for Clostridium difficile toxin B in the Cy5 channel:

16.4 Precision

The precision of the diarellaC.difficile real time PCR Kit was determined as intra-assay variability, inter-assay variability and inter-lot variability.
Variability data are expressed by standard deviation and coefficient of variation. The data are based on quantification analyses of defined concentrations of Clostridium difficile toxin A specific synthetic DNA, Clostridium difficile toxin B specific synthetic DNA and on the threshold cycle of the Control DNA (IPC). The results are shown in Table 9.

Table 9: Precision of the diarellaC.difficile real time PCR Kit

tcdA (FAM)	copies/µl	Standard Deviation	Coefficient of Variation (%)
Intra-Assay Variability	25	0.24	0.76
Inter-Assay-Variability	25	0.14	0.43
Inter-Lot-Variability	25	0.03	0.08
tcdB (Cy5)	copies/µl	Standard Deviation	Coefficient of Variation (%)
Intra-Assay Variability	25	0.10	0.32
Inter-Assay-Variability	25	0.48	1.56
Inter-Lot-Variability	25	0.19	0.63
IPC (HEX)	copies/µl	Standard Deviation	Coefficient of Variation (%)
Intra-Assay Variability	250	0.17	0.59
Inter-Assay-Variability	250	0.15	0.55
Inter-Lot-Variability	250	0.06	0.22

16.5 Diagnostic Sensitivity

The diagnostic sensitivity of real time (RT-) PCR assays is mainly dependent on the DNA/RNA extraction method used to isolate DNA and RNA from various biological specimens. DNA/RNA extraction reagents are not part of the gerbion real time (RT-) PCR kits. gerbion real time (RT-) PCR kits include an extraction control and guidelines for the validation criteria of the extraction control in each reaction. The extraction control indicates inhibition of the real time (RT-) PCR and/or inefficient nucleic acid extraction. It cannot be used as a calibrator.
Therefore, gerbion guarantees the analytical sensitivities and specificities of the real time (RT-) PCR kits, performed with eluted DNA and RNA from reference materials and ring trial samples and with synthetic nucleic acid fragments. gerbion does not guarantee diagnostic sensitivities. If diagnostic

sensitivities are mentioned in manuals of gerbion real time (RT-) PCR kits, the data are strictly correlated to a specific nucleic acid extraction method that has been used during the validation of the respective kits and cannot be transferred to other extraction methods. It is the responsibility of the user to qualify the extraction methods used for DNA/RNA isolation from biological samples.

17 Abbreviations and Symbols

DNA	Deoxyribonucleid Acid	REF	Catalog number
PCR	Polymerase Chain Reaction		Contains sufficient for <n> test
REACTION MIX	Reaction Mix		Upper limit of temperature
CONTROL +	Positive Control		Manufacturer
CONTROL −	Negative Control		Use by YYYY-MM-DD
CONTROL DNA IPC	Control DNA (IPC)	LOT	Batch code
CONT	Content	IVD	*In vitro* diagnostic medical device
	Consult instructions for use	CE	European Conformity
UDI	Unique Device Identification		

18 Literature

[1] Czepiel et al. (2019) Clostridium difficile infection: review. European Journal of Clinical Microbiology and Infectious Diseases. 38:1211-1221