Instruction for Use

diarellaBordetella

real time PCR Kit

For qualitative *in-vitro* detection of DNA of *Bordetella pertussis* and *Bordetella parapertussis* in clinical specimens.

REF	G01109-32	G01109-96
Σ	32	96

- 18°C

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1 Intended Use

The diarellaBordetella real time PCR Kit is an assay for the detection of DNA of *Bordetella pertussis* and *Bordetella parapertussis* in clinical specimens (respiratory samples) using real time PCR microplate systems.

2 Pathogen Information

B. pertussis, B. parapertussis, and B. bronchiseptica are the 3 important human pathogens that belong to the genus Bordetella. B. bronchiseptica can cause infectious bronchitis in dogs and other animals, but rarely infects humans. Only B pertussis produces the pertussis toxin (PT). B. parapertussis has been reported to cause whooping cough either as a single infective agent or with coinfection with B pertussis in almost 40% of laboratory-confirmed cases.

Pertussis (also known as whooping cough or 100-day cough) is a highly contagious bacterial disease. Initially, symptoms are usually similar to those of the common cold with a runny nose, fever, and mild cough. This is then followed by weeks of severe coughing fits. Pertussis is caused by the bacterium Bordetella pertussis.It is an airborne disease which spreads easily through the coughs and sneezes of an infected person. People are infectious to others from the start of symptoms until about three weeks into the coughing fits. Those treated with antibiotics are no longer infectious after five days. Diagnosis is by collecting a sample from the back of the nose and throat. This sample can then be tested by either culture or by polymerase chain reaction.

3 Principle of the Test

The diarellaBordetella real time PCR Kit contains specific primers and dual-labeled probes for the amplification and detection of DNA of *Bordetella pertussis* and *Bordetella parapertussis* in clinical specimens.

The presence of nucleic acid is detected by an increase in fluorescence due to hydrolysis of the probes during amplification. The emitted fluorescence is measured in the FAM (*Bordetella pertussis)* and ROX (*Bordetella parapertussis*) channel.

Furthermore, the diarellaBordetella real time PCR Kit contains a Control DNA, which is added during DNA extraction and detected in the same reaction by a differently labeled probe.

The Control DNA allows the detection of PCR inhibition and acts as control, that the nucleic acid was isolated from the clinical specimen. The fluorescence of the Control DNA is measured in the VIC®/HEX/JOE™/TET channel.

Note: The diarellaBordetella real time PCR Kit is designed to detect ≤10 genome copies per reaction by amplification of conserved multicopy regions. Therefore, cross-reactivities with B. bronchiseptica (B. parapertussis) and B. holmesii (B. pertussis) may occur.

4 Package Contents

The reagents supplied are sufficient for 32 or 96 reactions respectively.

Table 1: Components of the diarellaBordetella real time PCR Kit.

Label	Lid Colour	Content	
		32	96
Reaction Mix	yellow	1 x 512 µl	2 x 768 µl
Positive Control	red	1 x 50 µl	1 x 100 µl
Negative Control	green	1 x 50 µl	1 x 100 µl
Control DNA	colourless	1 x 160 µl	2 x 240 µl

5 Equipment and Reagents to be Supplied by User

- DNA isolation kit (e.g. NukEx Pure RNA/DNA, gerbion Cat. No. G05004 or 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
- Optional: Liquid handling system for automation
- Optional: BLP-DNA (Bacteria Like Particles, please look at chapter ‚Control DNA' for details)

6 Transport, Storage and Stability

The diarellaBordetella real time PCR Kit is shipped on dry ice or cool packs.
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.

For convenience, opened reagents can be stored at +2-8°C for up to 6 months. Protect kit components from direct sunlight during the complete test run.

7 Important Notes

- The diarellaBordetella real time PCR must be performed by qualified personnel only.
- Good Laboratory Practice (GLP) has to be applied.
- Clinical samples must always be regarded as potentially infectious material and all equipment used has to be treated as potentially contaminated.

8 General Precautions

- Stick to the protocol described in the Instruction for Use.
- Set up different laboratory areas for the preparation of samples and for the set up of the PCR in order to avoid contaminations.
- Pipettes, tubes and other materials must not circulate between those different laboratory areas.
- Always use filter tips.
- Regulary decontaminate equipment and benches with ethanol-free decontaminant.
- Do not combine diarellaBordetella real time PCR Kit components of different lot numbers.

9 Sample Material

Starting material for the diarellaBordetella real time PCR is the nucleic acid isolated from clinical specimens (respiratory samples).

10 Sample Preparation

The diarellaBordetella real time PCR is suitable for the detection of *Bordetella pertussis* and *Bordetella parapertussis* in clinical specimens (respiratory samples) isolated with suitable isolation methods.

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

- NukEx Pure RNA/DNA, gerbion Cat. No. G05004
- NukEx Mag RNA/DNA, gerbion Cat. No. G05012

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.

11 Control DNA

A Control DNA is supplied and can be used as extraction control or only as inhibition control. This allows the user to control the DNA isolation procedure and to check for possible real time PCR inhibition.
The Bacterium-Like Particles (BLP-DNA) are not supplied.

DNA isolation from clinical specimens

a) Control DNA or BLP-DNA used as Extraction Control:
diarellaBordetella real time PCR Control DNA or BLP-DNA is added to the DNA extraction.
Add 5 µl Control DNA or BLP-DNA per extraction (5 µl x (N+1)). Mix well. Perform the DNA isolation according to the manufacturer's instructions. Please follow protocol A.

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

b) Control DNA used as Internal Control of the real time PCR:
If only inhibition will be checked please follow protocol B.

12 Real time PCR

12.1 Important Points Before Starting:

- Please pay attention to the ‚Important Notes' on page 4.
- Before setting up the real time PCR familiarise 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 a Positive Control and one Negative Control should be included.
- Before each use, all reagents should be thawed completely at room temperature, thoroughly mixed (do NOT vortex the Reaction Mix but mix by pipetting up and down repeatedly), and centrifuged very briefly.

12.2 Procedure

If the Control DNA or BLP-DNA is used to control both, the real time PCR and the DNA isolation procedure, please follow protocol A. If the Control DNA is solely used to detect possible inhibition of the real time PCR, please follow protocol B.

Protocol A

The Control DNA or BLP-DNA was added during DNA extraction (see chapter ‚Control DNA'). In this case, prepare the Master Mix according to Table 2.

The Master Mix contains all of 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 (Control DNA was added during DNA extraction)

Volume per Reaction	Volume Master Mix
16.0 µl Reaction Mix	16.0 µl x (N+1)

Protocol B

The Control DNA is used for the control of the real time PCR only (see chapter ‚Control DNA'). In this case, prepare the Master Mix according to Table 3.

The Master Mix contains all of the components needed for real 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 3: Preparation of the Master Mix (Control DNA is added directly to the Master Mix)

Volume per Reaction	Volume Master Mix
16.0 µl Reaction Mix	16.0 µl x (N+1)
0.5 µl Control DNA*	0.5 µl x (N+1)*

*The increase in volume caused by adding the Control DNA is not taken into account when preparing the PCR assay.

Protocol A and B: real time PCR set up

- Place the number of optical PCR reaction tubes needed into the respective tray of the real time PCR instrument.
- Pipet **16 µl** of the Master Mix into each optical PCR reaction tube.
- Add **4 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 (Table 4).
- Close the optical PCR reaction tubes immediately after filling in order to reduce the risk of contamination.

Table 4: Preparation of the real time PCR

Component	Volume
Master Mix	16.0 µl
Sample	4.0 µl
Total Volume	20.0 µl

12.3 Instrument Settings

For the real time PCR use the thermal profile shown in Table 5.

Table 5: real time 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 Aquisition at the end of this step	60°C	

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

Table 6: Overview of the instrument settings required for the diarellaBordetella real time PCR Kit.

Real time PCR Instrument	Parameter	Detection Channel	Notes		
LightCycler 480I	*B. pertussis*	483-533	Colour Compensation Kit Multiplex 1 (G07OMP1-cc) required		
	B. parapertussis	558-610			
	Control DNA	523-568			
	-	615-670			
LightCycler 480II			Colour Compensation Kit Multiplex 1 (G07OMP1-cc) required		
			Melt Factor	Quant Factor	Max Integration Time (sec)
	B. pertussis	FAM (465-510)	1	10	1
	B. parapertussis	ROX (533-610)	1	10	2
	Control DNA	HEX (533-580)	1	10	2
	-	CY5 (618-660)	1	10	3
Stratagene Mx3000P / Mx3005P	*B. pertussis*	FAM	Gain 8		
	B. parapertussis	ROX	Gain 1		Reference Dye: None
	Control DNA	HEX	Gain 1		
	-	Cy5	Gain 4		
ABI 7500	*B. pertussis*	FAM	Option Reference Dye ROX: NO		
	B. parapertussis	ROX			
	Control DNA	JOE			
	-	Cy5			
Rotor-Gene Q, Rotor-Gene 3000 Rotor-Gene 6000	*B. pertussis*	Green	Gain 5		
	B. parapertussis	Orange	Gain 5		
	Control DNA	Yellow	Gain 5		
	-	Red	Gain 5		

13 Data Analysis

The *Bordetella pertussis* specific amplification is measured in the FAM channel and the *Bordetella parapertussis* specific amplification in the ROX channel.

The amplification of the Control DNA is measured in the VIC®/HEX/JOE™/TET channel.

Following results can occur:

- A signal in the FAM or ROX channel is detected:
  The result is positive, the sample contains bacterial DNA.
  In this case, detection of a signal of the Control DNA in the VIC®/HEX/JOE™/TET channel is inessential, as high concentrations of bacterial DNA may reduce or completely inhibit amplification of the Control DNA.
- No signal in the FAM or ROX channel, but a signal in the VIC®/HEX/JOE/TET channel is detected:
  The result is negative, the sample does not contain bacterial DNA.
  The signal of the Control DNA excludes the possibilities of DNA isolation failure (in case the Control DNA is being used as an Extraction Control) and/or real time PCR inhibition. If the C_T value of a sample differs significantly from the C_T value of the water control, a partial inhibition occured, which can lead to negative results in weak positive samples (see 'Troubleshooting').
- Neither in the FAM and ROX channel nor in the VIC®/HEX/JOE/TET channel a signal is detected:
  A diagnostic statement cannot be made.
  The DNA isolation was not successful or an inhibition of the PCR has occurred. In case the Control DNA was added during DNA isolation and not directly to the PCR Master Mix, the Negative Control is negative in both channels.

Note: The diarellaBordetella real time PCR Kit is designed to detect ≤10 genome copies per reaction by amplification of conserved multicopy regions. Therefore, cross-reactivities with B. bronchiseptica (B. parapertussis) and B. holmesii (B. pertussis) may occur.

Figure 1 and **Figure 2** show examples for positive and negative real time PCR results.

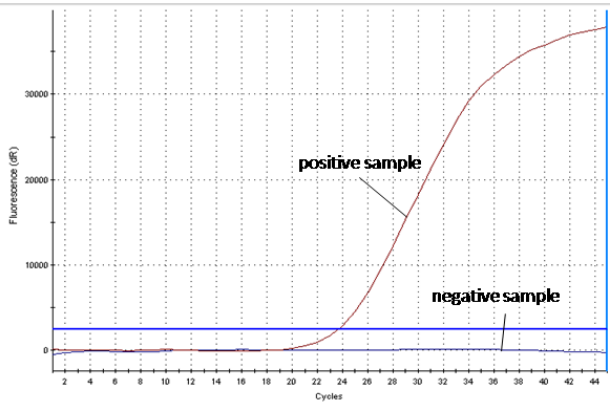

Figure 1: The positive sample shows bacteria specific amplification in the FAM channel, whereas no fluorescence signal is detected in the negative sample.

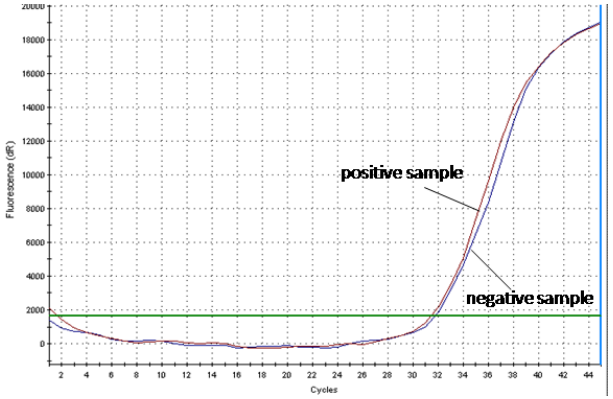

Figure 2: The positive sample as well as the negative sample show a signal in the Control DNA specific VIC®/HEX/JOE™/TET channel. The amplification signal of the Control DNA in the negative sample shows, that the missing signal in the bacteria specific FAM channel is not due to PCR inhibition or failure of DNA isolation, but that the sample is a true negative.

14 Assay Validation

Set a threshold as follows:

Negative Controls

All negative controls should be below the threshold. If there is a potential contamination (appearance of a curve in the negative control or a cluster of curves in specimens at high C_T – for example above 36), results obtained are not interpretable and the whole run (including extraction) has to be repeated.

Positive Controls

All the positive controls must show a positive (i.e. exponential) amplification curve. The positive controls must fall below a C_T of 30.

Internal Controls

All internal controls must show a positive (i.e. exponential) amplification curve. The internal control must fall below a C_T of 33. If the internal control is above C_T 34, this points to a purification problem or a strong positive sample that can inhibit the IC. In the latter case, the assay is valid. If a water control run is performed, the IC must fall below a C_T of 33.

15 Limitations of the Method

The results must always be considered in relation to the clinical symptoms. Therapeutical consequences should be made in consideration of clinical data.
A negative test result does not exclude a *Bordetella pertussis* or *Bordetella parapertussis* infection.

16 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 or ROX channel of the Positive Controls	
The selected channel for analysis does not comply with the protocol	Select the FAM channel for analysis of the Bordetella pertussis specific amplification, the ROX channel for analysis of the Bordetella parapertussis specific amplification and the VIC®/HEX/JOE™/TET channel for the amplification of the Control DNA. Due to amplification both specific channels, amplification of the Internal Control can be inhibited in the Positive Control.
Incorrect configuration of the real time PCR	Check your work steps and compare with ‚Procedure' on page 7.
The programming of the thermal profile is incorrect	Compare the thermal profile with the protocol (Table 5, page 9).
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 ‚Transport, Storage and Stability', page 4.
Weak or no signal of the Control DNA and simultaneous absence of a signal in the bacteria specific FAM channel or ROX channel.	
real time PCR conditions do not comply with the protocol	Check the real time PCR conditions (page 6).
real time PCR inhibited	Make sure that you use an appropriate isolation method (see ‚Sample Preparation', page 5) and follow the manufacturer's instructions. Make sure that the ethanol-containing washing buffer of the isolation kit has been completely removed. An additional centrifugation step at high speed is recommended before elution of the DNA.
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 ‚Transport, Storage and Stability', page 4.

Detection of a fluorescence signal in the FAM channel or ROX 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 occured 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.

17 Kit Performance

17.1 Diagnostic Sensitivity and Specificity

During the validation study of the diarellaBordetella real time PCR ring trial samples were tested. The table below shows the outcome of the ring trial.

Table 7: Results of Bordetella ring trial.

Sample	Sample Content	Expected Result	Result diarellaBordetella	Sample Type
BPDNA14-01	Bordetella negative	negative	negative	core
BPDNA14-02	B. pertussis (BORD945)	positive	positive	educational
BPDNA14-03	B. pertussis (BORD945)	positive	positive	core
BPDNA14-04	B. pertussis	positive/negative	negative	educational
BPDNA14-05	B. pertussis	positive	positive	core
BPDNA14-06	B. pertussis	positive	positive	educational
BPDNA14-07	B. pertussis	positive	positive	core
BPDNA14-08	B. parapertussis	positive	positive	educational
BPDNA14-09	H. influenzae	negative	negative	core
BPDNA14-10	B. bronchiseptica (IS481-)	negative	negative	educational
BPDNA14-11	B. holmesii (IS481+)	negative	positive	educational
BPDNA14-12	B. bronchiseptica (IS481+)	negative	positive	educational

17.2 Analytical Sensitivity

The limit of detection (LoD) of diarellaBordetella real time PCR was determined using serial dilutions of synthetic target DNA-sequences in a Stratagene Mx3000 real time PCR instrument. The LoD of diarellaBordetella real time PCR for *Bordetella pertussis and Bordetella parapertussis* is ≤10 target copies per reaction each.

17.3 Analytical Specificity

The specificity of diarellaBordetella real time PCR was determined by in silico analysis. The specificity was evaluated additionally with different other relevant viruses and bacteria found in clinical samples.

Results:

The diarellaBordetella real time PCR showed a positive result for the samples containing *B. pertussis and B. parapertussis*, whereas samples containing other pathogens were reliably tested negative. The results are shown in Table 8.

Table 8: Bacterial and viral pathogens tested for the determination of the analytical sensitivity of the diarellaBordetella real time PCR Kit.

Strain	Expected Result	Result
Adenovirus Serogroup 14	negative	negative
Adenovirus Serogroup 4	negative	negative
Echovirus 30	negative	negative
Echovirus 11	negative	negative
Haemophilus influenzae	negative	negative
Influenza A A/California/7/2009	negative	negative
Influenza A A/Sachsen/2/2015	negative	negative
Influenza A A/Switzerland/97 15293/2013	negative	negative
Influenza A A/Victoria/361/2011	negative	negative
Influenza B B/ Phuket/3073/2013	negative	negative
Influenza B B/Massachusetts/ 2/2012	negative	negative
Legionella longbeachae	negative	negative
Legionella pneumophila Serogroup 1	negative	negative
Metapneumovirus A1	negative	negative
Metapneumovirus A2	negative	negative
Metapneumovirus B2	negative	negative

Mycobacterium tuberculosis	negative	negative
Mycoplasma pneumoniae	negative	negative
Parainfluenza Type 1	negative	negative
Parainfluenza Type 2	negative	negative
Parainfluenza Type 3	negative	negative
Parainfluenza Type 4	negative	negative
Parechovirus 1	negative	negative
Parechovirus 2	negative	negative
Parechovirus 3	negative	negative
Parechovirus 4	negative	negative
Parechovirus 5	negative	negative
RSV A	negative	negative
RSV B	negative	negative
Bordetella parapertussis DSMZ, Type Strain 13415	positive	positive
Bordetella pertussis DSMZ, Type Strain 5571	positive	positive
B. holmesii	positive / negative	positive / negative
B. bronchiseptica	positive / negative	positive / negative

18 Abbreviations and Symbols

DNA	Deoxyribonucleid Acid	REF	Catalog number
PCR	Polymerase Chain Reaction	Σ	Contains sufficient for <n> test
REACTION MIX	Reaction Mix	- 18°C	Upper limit of temperature
CONTROL +	Positive Control		Manufacturer
CONTROL −	Negative Control		Use by YYYY-MM
CONTROL DNA IC	Control DNA		Consult Instructions for Use
LOT	Batch code	IVD	*In vitro* diagnostic medical device
CONT	Content	CE	European Conformity

19 Literature

[1] *Centers for Disease Control and Prevention. "Pertussis". The Pink Book. Retrieved 4 May 2016.*

[2] S. Swidsinski, G. Schmidt-Schläpfer, B. Schneeweiß, A. Swidsinski: *Pertussis-PCR Neuer Standard in der Keuchhustendiagnostik Auswertung eines ersten multizentrischen Ringversuchs in Deutschland und der Schweiz*. In: *Monatsschrift Kinderheilkunde*. 146, Nr. 12, 1998, S. 1171-1175. ISSN 0026-9298.