BactoReal® Kit *Bordetella* Multiplex

Kit version 1.1

Instructions for Use

CE

IVD **For *in vitro* diagnostic use**

REF **DHUB00453**

Σ **50 reactions**

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Explanation of symbols

LOT	Batch code		Use by date
REF	Catalogue number		Manufacturer
Σ	Contains sufficient for <n> tests		Temperature limit (Store at)
CE	This product fulfils the requirements of the European Directive 98/79 EC for *in vitro* diagnostic medical devices	IVD	In vitro diagnostic medical device
	Consult instructions for use	UDI	Unique device identifier
	Keep away from sunlight	QTY	Contents

1. Intended purpose

BactoReal® Kit *Bordetella* Multiplex is a non-automated IVD real-time PCR test for the qualitative detection and identification of DNA of *Bordetella pertussis, Bordetella parapertussis,* and *Bordetella holmesii*. This test detects the insertion sequence IS481 of *B. pertussis and B. holmesii* as well as the insertion sequence IS1001 of *B. parapertussis* and *B. holmesii*. IS481 or IS1001 may be present in *B. bronchiseptica* in rare cases.

Proper specimens are DNA extracts isolated from samples of the human respiratory tract (sputum, BAL, nasal swabs, throat swabs, bronchial secretion, tracheal secretion).

This test is suitable for patients of all ages with suspected respiratory infection with *Bordetella* (causative agent of whooping cough and whooping cough-like infections) and is intended as an aid in the diagnosis of infection with this pathogen in combination with patient history and additional clinical information.

The test is intended for professional use and is limited to qualified personnel instructed in the procedures of real-time PCR and *in vitro* diagnostic procedures.

2. Product description

BactoReal® Kit *Bordetella* Multiplex is a real-time PCR test and detects in the fluorescence channel for FAM the insertion sequence IS481 of *B. pertussis* and *B. holmesii* (multicopy gene, found up to 249 times in the genome of *B. pertussis*, Weigand et al., 2019) and in the fluorescence channel for VIC the insertion sequence IS1001 of *B. parapertussis* and *B. holmesii* (multicopy gene, found up to 22 times in the genome of *B. parapertussis*, Weigand et al., 2019). A probe-specific amplification-curve in the FAM channel and VIC channel indicates the amplification of *Bordetella holmesii* specific DNA.
Sometimes a few copies of IS481 or IS1001 may be present in B. bronchiseptica, which could possibly be detected if the concentration of bacteria is high.

The internal DNA positive control (IPC) is detected in the fluorescence channel Cy5 and serves as a control for DNA extraction and possible real-time PCR inhibition. The target for the DNA IPC (artificial target DNA) is added during sample extraction.

This test has been validated with the ABI® 7500 Fast Real-Time PCR System (fast cycle parameters are not supported, Thermo Fisher Scientific) and was also tested with a LightCycler® 480 II (Roche Diagnostics), QuantStudio™ 7 real-time PCR system (Thermo Fisher Scientific) and Mic instrument (bio molecular systems).
It is also compatible with other real-time PCR instruments which detect and differentiate fluorescence in FAM, VIC and Cy5 channel (e.g., QuantStudio™ 5 real-time PCR system (Thermo Fisher Scientific), qTOWER^{3}G (Analytik Jena), cobas z 480 Analyzer (Roche), CFX Opus Real-time PCR System (Bio-Rad), CFX Dx (Bio-Rad) and T-COR 8 (Tetracore)).

When using PCR-platforms not tested by ingenetix, an evaluation of the multiplex-PCR shall be done. Keep in mind that some PCR-platforms first must be calibrated with the corresponding dye before performing multiplex-PCR.

Ingenetix BactoReal®, ViroReal®, MycoReal®, PanReal and ParoReal® Kits are optimized to run under the same thermal cycling conditions. DNA and RNA can be analysed in one run.

3. Pathogen information

Bordetella pertussis is a gram-negative bacterium of the genus *Bordetella* and the causative agent of pertussis, an acute respiratory disease. Transmission occurs by droplet infection. *Bordetella parapertussis*, *Bordetella holmesii* and *Bordetella bronchiseptica* can cause a whooping cough-like illness with a milder clinical course. While *Bordetella pertussis* and *Bordetella parapertussis* only infect humans, *Bordetella bronchiseptica occurs* mainly in animals and is occasionally isolated from humans.

4. Principle of real-time PCR

The test is based on multiplex real-time PCR by 5´-nuclease-assay technology. Specific DNA sequences are amplified, and the generated PCR-products are detected by oligonucleotide-probes labelled with fluorescent dyes. This allows a sequence-specific detection of PCR amplificates.

During PCR, primers are extended by *Taq* polymerase and probes hybridized to the target are cleaved by the 5'-exonuclease activity of *Taq* polymerase. According to the accumulation of PCR product, the fluorescence of the probe increases with each PCR cycle. The change in fluorescence of the different dyes is recorded cycle by cycle in the real-time PCR instrument in the closed reaction tube at different fluorescence wavelengths.
The Cq value (Cq = Quantification cycle, Ct = Cycle threshold, Cp = Crossing point) describes the cycle at which the fluorescence first rises significantly above the background fluorescence.

5. Contents of the kit, stability, and storage

Table 1

Labelling	Content	Amount	Storage
Bordetella + IPC3 Assay Mix (green cap)	Primers and probes for detection of - *Bordetella* (FAM+VIC) - DNA IPC (Cy5)	1 x 50 µl	-25 to -15 °C
IPC-Target (DNA) (orange cap)	Target for DNA IPC (internal DNA positive control system)	1 x 200 µl	-25 to -15 °C
Bordetella Positive Control (red cap)	DNA positive control for *B. pertussis* + *B. parapertussis* (approx. 1000 target copies/µl)	1 x 300 µl	-25 to -15 °C
DNA Reaction Mix (white cap)	PCR reaction mix for DNA amplification	1 x 500 µl	**-25 to -15 °C after first opening 2 to 8 °C**
Nuclease-free water (blue cap)	Nuclease-free water	1 x 1000 µl	-25 to -15 °C

DNA Reaction Mix
The Master Mix provided with the kit has been designed for reliable, high-sensitivity real-time PCR. The Master Mix contains a highly purified Taq Polymerase for rapid hot-start PCR, dNTPs with dUTP and Uracil-N glycosylase (UNG) to eliminate amplicon carryover, ROX™ dye (passive reference) and buffer components – additives optimized to handle RT-PCR inhibitors.

Delivery and Storage
Shipment is with coolpacks or on dry ice. When stored properly, the kit components are stable until the specified expiration date. This also applies after opening. Store kit protected from light.

Quality Control Release Testing
In accordance with the ISO 13485 certified Quality Management System of ingenetix, each lot is tested against predetermined specifications to ensure consistent product quality. Quality control is performed with a plasmid containing parts of the pathogen DNA.

6. Additionally required materials and devices

- Reagents and devices for DNA-extraction which are appropriate for the listed sample material (see 9. Preparation of the samples)
- Nuclease-free water
- Powder-free disposable gloves
- Pipettes (adjustable)
- Filter pipette tips
- Vortex-Mixer
- Benchtop centrifuge with rotor for 2 ml reaction tubes
- Real-time PCR instrument detecting and differentiating fluorescence in FAM, VIC and Cy5 channel (see 2. Product description)
- Appropriate 96 well optical reaction plates or reaction tubes with corresponding (optical) closing material
- Optional: Laminar flow box
- Optional: PCR workstation

7. Precautions and safety information

7.1. General information

- For *in vitro* diagnostic use. The use of this kit is limited to qualified personnel instructed in real-time PCR and *in vitro* diagnostic procedures.
- Transportation of clinical specimens must comply with local regulations for the transport of biological materials.
- Specimens should be handled as if infectious in accordance with safe laboratory procedures. Wear protective powder-free disposable gloves when handling kit reagents and specimens.
- Discard specimens, reagents, and waste according to your local safety regulations.
- Improper collection, transport or storage of specimens may hinder the ability of the assay to detect the target sequences.
- Quality of DNA has a profound impact on the test performance. Ensure that the used DNA extraction system is compatible with real-time PCR technology.
- The real-time PCR instrument should be calibrated, serviced, and cleaned regularly.
- Protect kit components from light.
- Avoid mixing reagents of different kits and lots and check expiry date of the kit.

7.2. Specific information

A workflow must be followed to avoid false positive results due to detection of contaminating DNA.

<u>Recommended measures to avoid DNA contamination:</u>

- Use separated workspaces for specimen preparation, set-up of real-time PCR and amplification. Supplies and equipment must be dedicated to each of these separate workspaces to ensure workflow in the laboratory from pre- to post-PCR.
- Laboratory benches and devices must be cleaned regularly.
- Sample preparation should be performed in a laminar flow box. Clean laminar flow box regularly in all areas.
- If possible, leave consumables and pipettes in the laminar flow sterile bench and PCR workstation.
- Preparation of real-time PCR should be done in a PCR workstation.
- The use of sterile aerosol-resistant pipette tips is required.
- Use only DNA-free consumables.
- Wear lab coat.
- Work only with powder-free disposable gloves, do not touch the outer surface of the palm and fingers of the gloves when putting them on. Change gloves frequently. To avoid skin contact, wear gloves over the sleeves of the lab coat. Use disposable sleeve protectors if possible.
- Do not touch the rim or threads of open vials.
- Caution is advised when handling specimens and positive control to avoid cross-contamination.
- Store positive and potentially positive material separately from all other reagents.
- For a valid interpretation of results, a negative control shall be included during DNA-extraction (e.g., extraction of water instead of sample material), in to exclude false-positive results due to contamination with DNA of pathogen during extraction.
- Optional: include a negative control of PCR per PCR-run (nuclease-free water instead of sample, NTC).

8. Limitations

- Reliable results with this test are only achieved by appropriate specimen collection, transport, and storage, as well as an appropriate DNA extraction procedure.
- DNA extraction and *Bordetella* detection have been validated for respiratory specimens with this kit.
- A negative test result does not exclude the possibility of a *Bordetella* infection, because test results may be affected by improper specimen collection, technical error, specimen mix-up or pathogen quantities below the assay sensitivity. PCR inhibitors may lead to invalid results.
- For this kit highly specific primers and probes have been selected. However, false-negative, or less sensitive results might be obtained due to sequence heterogeneity within the target region of yet unknown clinical subtypes.
- Results should be interpreted in context of other clinical and laboratory findings.

9. Preparation of samples

BactoReal® Kit *Bordetella* Multiplex is suitable for analysis of DNA extracts of samples from the human respiratory tract (sputum, BAL, nasal swabs, throat swabs, bronchial secretion, tracheal secretion).

Sample preparation should be performed with the recommended measures to avoid DNA contamination (see precautions and safety information). Always include a DNA extraction negative control (e.g., extraction of water).

Purified DNA should be stored at -25 to -15 °C.

9.1. Sample collection and storage

Samples from the respiratory tract can be stored in microcentrifuge tubes.
Swabs can be stored dry or put into 1 ml isotonic saline solution (NaCl 0.9%, not provided). Swab material proper for PCR: e.g., sterile polyester or rayon swabs with plastic or aluminium shaft.
It is recommended to process samples immediately after collection. Store samples at 2-8 °C for no longer than 48 hours or freeze at -20/-80 °C.

9.2. Recommended extraction methods

Ensure that the extraction system used is not contaminated with DNA of pathogens detected with BactoReal® Kit *Bordetella* Multiplex. Extract the sample using a DNA extraction system compatible with real-time PCR technology and appropriate for the sample material.

<u>For manual extraction recommended</u>
- QIAamp DNA Mini Kit (Qiagen)
- High Pure PCR Template Preparation Kit (Roche)

<u>For automated extraction recommended</u>
- innuPREP AniPath DNA RNA – KFFLX Kit (Analytik Jena) with the KingFisher FLEX instrument (Thermo Fisher Scientific)
- MagNA Pure 96 DNA and Viral NA Small Volume Kit with the Roche MagNA Pure Compact or Roche MagNAPure 96 System.

When using extraction methods not recommended by ingenetix, an evaluation of the extraction method must be performed.

9.3. Quality control for DNA extraction and PCR inhibition with IPC

The DNA IPC system (internal DNA positive control) is used as a control for DNA extraction, identifies possible PCR inhibition and confirms the integrity of kit reagents.
For this, an artificial target DNA (IPC-Target (DNA), approx. $6x10^5$ copies/µl) is added during extraction.

Note: The Cq values of the IPC depend on the extraction method and on the type of sample material. Negative samples should show Cq values of the IPC between 27-30. The extraction method used must be validated accordingly with sample material. Use the IPC-Target (DNA) freshly diluted (1:10 with nuclease-free water) in the extraction, if Cq values < 27 are determined during validation with samples.

9.3.1. Application of IPC as control of extraction and real-time PCR

The IPC-Target (DNA) must be added during extraction.

→ Per sample spike <u>1 µl IPC-Target (DNA)</u> (orange cap) directly into the appropriate volume of lysis buffer (or spike it to the sample <u>after</u> the lysis buffer has been added to the sample), then continue the extraction procedure.

Caution: The IPC-Target (DNA) shall not be added to sample material in the absence of lysis buffer, as degradation may occur. It must be added to the lysis buffer.

9.3.2. Application of IPC as quality control for PCR reaction

If the IPC-Target (DNA) has not been added during extraction, it can be added at a later stage to the PCR master mix.

→ Freshly dilute the IPC-Target (DNA) 1:100 with nuclease-free water and add 1 µl of the dilution/PCR reaction (approx. 6000 target copies).

Caution: The IPC-Target (DNA) shall not be added to the master mix undiluted.

10. Preparation of real-time PCR

- Include one positive control (red cap), one extraction negative control and optionally one PCR negative control (NTC, e.g., nuclease-free water) per PCR run.
- It is generally recommended to analyse samples in duplicates, which increases the probability of pathogen detection and facilitates interpretation of results.
- Thaw DNA samples on ice.
- Thaw kit components completely at room temperature. When thawed, mix components carefully, centrifuge briefly with low speed.
- Mix the DNA Reaction Mix gently to ensure homogeneity of solution.
- **Positive Control**
  → Use 9 µl of Positive Control (red cap). Always, pipette positive control at last.

10.1. Pipetting scheme

		Per sample
Preparation of Master Mix (mix well)	DNA Reaction Mix	10.0 µl
	Bordetella + IPC3 Assay Mix	1.0 µl
	Total volume Master Mix	**11.0 µl**
Preparation of PCR	Master Mix	11.0 µl
	DNA-Sample*	9.0 µl
	Total volume	**20.0 µl**

*1-9 µl of the sample can be used. For ≠ 9 µl sample, the volume must be adjusted with nuclease-free water.

→ **If IPC-Target (DNA) was not added during extraction:**
Freshly dilute the IPC-Target (DNA) (orange cap) 1:100 with nuclease-free water and add 1 µl per sample directly to the master mix. In this case, the IPC is used for quality control of the PCR reaction. Only 8 µl of DNA sample can be analyzed.
Caution: The use of more than 1 µl diluted IPC-Target (DNA) per reaction might cause inhibition of the real-time PCR reaction.

- Prepare the Master Mix according to the number of samples, calculating an additional volume of approx. 10% for pipetting loss.
- Pipette 11 µl of the prepared Master Mix per sample into the well of the optical reaction plate.
- Then add 9 µl of the extracted sample or controls. Pipette the positive control at last.
- Seal the plate with a suitable optical sealing material.
- Vortex the sealed plate for 1-2 seconds and briefly centrifuge the plate.

10.2. Programming of temperature profile

Please find further information on programming of the real-time PCR instrument in the respective operator's manual. Keep in mind that some PCR-platforms must be calibrated with the corresponding dye before performing a multiplex-PCR.

Sample Volume: 20 µl
Ramp speed: "Standard" without "fast cycling" parameter for ABI® 7500 Fast, QuantStudio™ (Thermo Fisher Scientific)

Program 1 Cycles: 1 Analysis: None	**Program 2** Cycles: 1 Analysis: None	**Program 3** Cycles: 45 Analysis: Quantification Acquisition at 60 °C
50 °C 2 min	95 °C 2 min	95 °C 5 sec 6 0°C 1 min

Ad program 1: If viral RNA should be also detected in the same PCR run, program 1 must be prolonged to 15 min at 50 °C. This temperature profile can be used for all ingenetix BactoReal®, ViroReal®, MycoReal®, PanReal and ParoReal® kits for the detection of DNA or RNA.
Ad program 2: the previous temperature profile with 20 sec in program 2 can still be used.

<u>Select detection channels</u>

FAM channel: Detection of *B. pertussis, B. holmesii, (B. bronchiseptica)*
VIC channel: Detection of *B. parapertussis, B. holmesii*, *(B. bronchiseptica)*
Cy5 channel: Detection of IPC

For ABI® 7500 Instrument, QuantStudio™ 5/6/7 (Thermo Fisher Scientific), Mx3005P® (Agilent)
FAM-NONE
VIC-NONE
Cy5-NONE
Passive refence dye: ROX

For MIC Instrument (bio molecular systems)
FAM Green
VIC Yellow
Cy5 Red
Passive refence dye: no ROX needed

For cobas z 480 Analyzer / LightCycler® 480 II (Roche)
Detection format: 3 Color Hydrolysis Probe
FAM: Excitation at 465 nm, Emission at 510 nm
VIC: Excitation at 540 / 533 nm, Emission at 580 nm
Cy5: Excitation at 610 / 618 nm, Emission at 670 / 660 nm
Passive reference dye: no ROX needed

The color compensation for FAM and VIC must be selected from the Roche database after the analysis of Cy5.

11. Interpretation of data

For the analysis of the PCR results, select the fluorescence display options FAM and VIC channel for the pathogen target and Cy5 channel for the IPC.
Please note that some PCR platforms require Color Compensation when using a multiplex PCR.

IMPORTANT: Samples should be checked in both logarithmic (Roche instrument: Abs Quant/Fit Points) and linear view and compared to the negative controls. In addition to the Cq values, also check the amplification curves and adjust the threshold (noise band) if necessary.
After you have saved the new settings, export the data. For the cobas z 480 Analyzer, export tables per dye.

Assessment of clinical specimen test results should be performed after the positive and negative controls have been examined and determined to be valid. If results of controls are not valid, no interpretation of results with clinical samples is possible.

Negative samples:
Samples without amplification curves (no Cq values, undetermined) are regarded as negative. No DNA was detected in these samples because there is no infection with the corresponding pathogen, or the pathogen DNA concentration is below the detection limit of the test.

Positive samples:
Samples with Cq values < 45 in the fluorescence channel for the pathogen target are regarded as positive.

Table 2 shows the criteria for valid positive and negative controls. Table 3 shows interpretation of data with clinical samples.

11.1. Controls

Table 2 Criteria for valid controls, IPC-Target (DNA) was added during extraction

	Cq FAM channel IS481 *B. pertussis*	Cq VIC channel IS1001 *B. parapertussis*	Cq Cy5 channel IPC[1]	Interpretation	Action
Positive control	< 32	< 30	Negative	Valid	-
Positive control	Negative	Negative	Negative	Invalid	See 12.1
Positive control	< 32	Negative	Negative	Invalid	See 12.1
Positive control	Negative	< 30	Negative	Invalid	See 12.1
Extraction negative control	Negative	Negative	27-30[2]	Valid	-
Extraction negative control	Negative	Negative	Negative	Invalid	See 12.1
Extraction negative control	Positive	Positive	27-30[2]	Invalid	See 12.3
Extraction negative control	Positive	Negative	27-30[2]	Invalid	See 12.3
Extraction negative control	Negative	Positive	27-30[2]	Invalid	See 12.3
NTC PCR (optional)	Negative	Negative	Negative	Valid	-
NTC PCR (optional)	Positive	Positive	Negative	Invalid	See 12.2
NTC PCR (optional)	Negative	Positive	Negative	Invalid	See 12.2
NTC PCR (optional)	Positive	Negative	Negative	Invalid	See 12.2

[1] If the IPC-Target (DNA) was added directly to the master mix, all samples in the Cy5 channel must be positive.
[2] Cq values of the IPC are dependent on the extraction method and should show Cq values of 27-30 (see 9. preparation of samples).

11.2. Clinical samples

Samples with positive Cq-values are interpreted as positive, see Table 3.

Table 3 Interpretation of data with clinical samples

	Cq FAM channel IS481	Cq VIC channel IS1001	Cq Cy5 channel IPC[1]	Interpretation	Action
Clinical sample	Negative	Negative	27-30	Negative	-
Clinical sample	Positive	Negative	Positive/Negative[2]	Positive for *B. pertussis*	-
Clinical sample	Negative	Positive	Positive/Negative[2]	Positive for *B. parapertussis*	-
Clinical sample	Positive	Positive[3]	Positive/Negative[2]	Positive for *B. holmesii*	-
Clinical sample	Positive/Negative	Positive/Negative	Positive/Negative[2]	Positive for *B. bronchiseptica*[4]	
Clinical sample	Negative	Negative	Negative	Invalid	See 12.5

[1] A positive signal of the IPC excludes potential PCR inhibition. However, IPC Cq-values should show comparable results among samples. A shift of Cq- values can indicate a partial inhibition of PCR. Cq values of the IPC are dependent on the extraction method and should show Cq values of 27-30 (see 9. preparation of samples).
[2] High pathogen loads in the sample can lead to a reduced or absent fluorescence signal of the IPC.
[3] If the fluorescence of the amplification curve in the VIC channel is significantly lower than in the FAM channel, *B. holmesii* is detected. See Figure 1.
[4] If a high pathogen concentration is present, *B. bronchiseptica* IS481 or IS1001 could possibly also be detected.

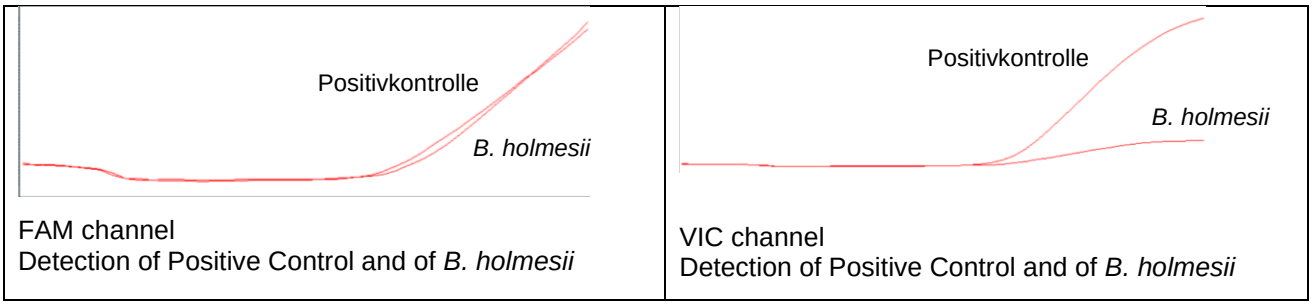


Figure 1: linear view of the amplification curve

In case of invalid data, analysis must be repeated with the remaining or newly extracted DNA sample (see 12. Troubleshooting).

12. Troubleshooting

12.1. No pathogen specific signal with positive control and with IPC

- Incorrect programming of the temperature profile or incorrect setting of detection channels on the real-time PCR instrument.
  → Compare temperature profile and setting of detection channels with details specified in the protocol
- Incorrect configuration of PCR reaction.
  → Check your pipetting steps with the pipetting scheme and repeat PCR, if necessary.
  → The DNA may have been degraded.
- The IPC-Target (DNA) was added undiluted directly to the master mix and not freshly diluted 1:100. The PCR reaction is therefore inhibited.
  → Freshly dilute IPC-Target (DNA) 1:100 and repeat PCR.
- No Positive Control was added.
  → Repeat PCR in case all clinical samples are negative.
- For control of real-time PCR only: 1 µl of freshly 1:100 diluted IPC-Target (DNA) must be added to the master mix. If the addition of IPC-Target has been forgotten:
  → Freshly dilute IPC-Target (DNA) and repeat PCR.
- For control of DNA extraction and PCR inhibition, the undiluted IPC-Target (DNA) must be added during extraction to the lysis buffer. If the addition of IPC-Target has been forgotten:
  → Repeat DNA extraction.

12.2. Pathogen signal with negative control

- If there are Cq values in the negative control below the cut-off specified in the Instructions for Use, a contamination occurred during the preparation of the PCR.
  → Repeat PCR with new reagents in replicates.
  → Strictly pipette positive control at last.
  → Make sure that workspace and instruments are cleaned at regular intervals.

12.3. Pathogen signal with extraction negative control

- If there are Cq values in the negative control of extraction below the cut-off specified in the Instructions for Use, a contamination occurred during the preparation of the PCR or the DNA extraction.
  → Repeat the PCR with unused reagents in replicates.
  → If repeatedly positive: Repeat DNA extraction and PCR using new reagents.
  → Strictly pipette positive control at last.
  → Make sure that workspace and instruments are cleaned at regular intervals and that workstations are separated from samples with high pathogen concentration.

12.4. IPC specific signal with PCR negative control and positive control

- The IPC-Target (DNA) has been added to lysis buffer during extraction, but there is IPC specific signal with PCR-NTC and positive control: Contamination with the IPC-Target (DNA).
  → Make sure that workspace and instruments are cleaned at regular intervals.

12.5. No signal with IPC and no pathogen specific signal with sample

- Incorrect assignment of detection channels in sample.
  → Please verify the correct assignment of detection channels.
- The DNA might be degraded.
- If the IPC-Target (DNA) was added during extraction:
  - Inhibition of PCR may have occurred.
  - DNA extraction was unsuccessful.
  - The IPC-Target (DNA) was not added to the lysis buffer of the sample.
  - The extracted sample was not added to the PCR-reaction.

  → No statement is possible. Verify you use a recommended method for DNA isolation and re-examine the single steps of the DNA extraction.
  → If no operating mistakes during DNA extractions can be retraced, it is recommended to repeat the PCR with lower amounts of DNA-eluate (1/5 or 1/10 of sample volume + the adequate volume of nuclease-free water).

13. Specifications and performance evaluation

13.1. Test performance on different real-time PCR instruments

Performance of BactoReal® Kit *Bordetella* Multiplex with an ABI® 7500 instrument (Thermo Fisher Scientific) is shown in Figure 2.

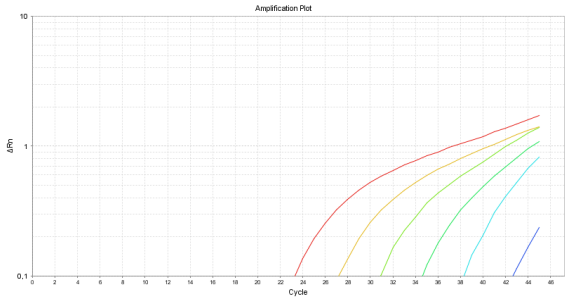
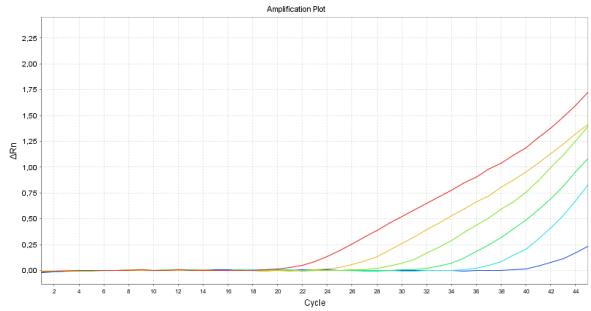
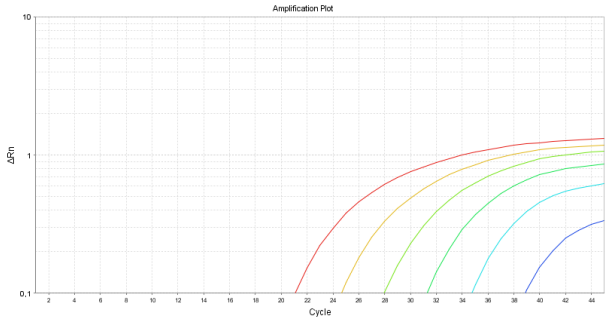
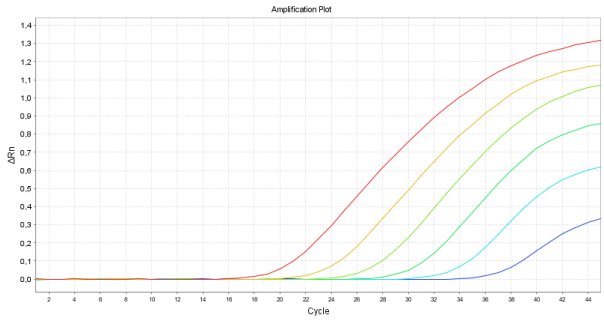
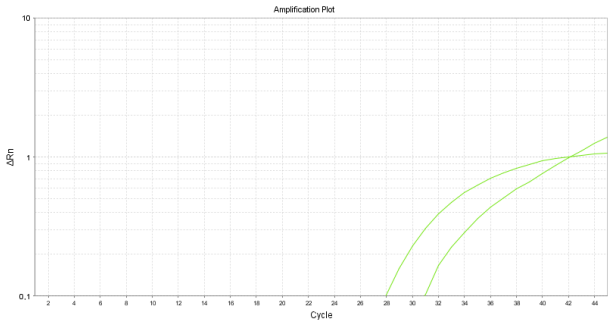
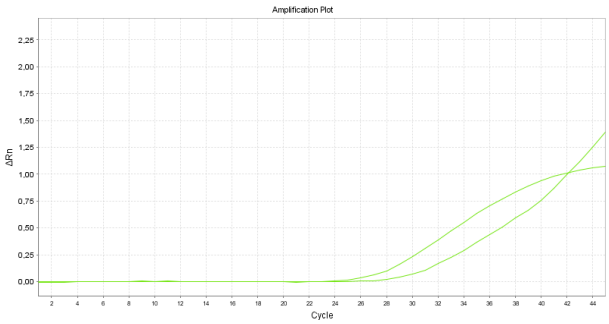
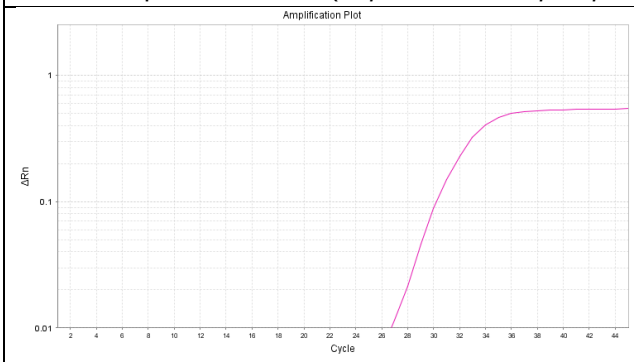
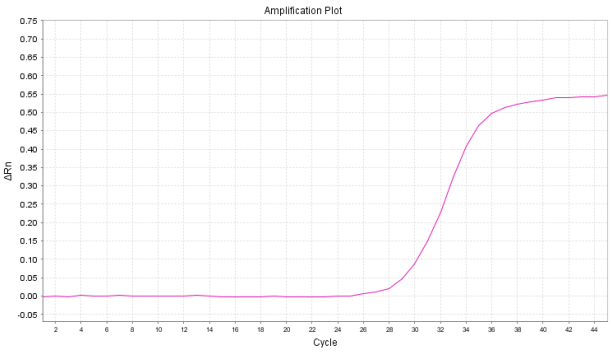
Logarithmic view	Linear view
	
ABI® 7500: FAM channel 1:10 serial dilution of a *B. pertussis* DNA target (E+6 to E+1 copies)	
	
ABI® 7500: VIC channel 1:10 serial dilution of a *B. parapertussis* DNA target (E+5 to E+0 copies)	
	
ABI® 7500: FAM and VIC channel *Bordetella* positive control (*B. pertussis* + *B. parapertussis)* (E+4 copies)	
	
ABI® 7500: Cy5 channel Internal DNA positive control	

Figure 2 Performance of BactoReal® Kit *Bordetella* Multiplex

This test has been validated with the ABI® 7500 instrument (Thermo Fisher Scientific) and was also tested with a LightCycler® 480 II (Roche Diagnostics), QuantStudio™ 7 real-time PCR system (Thermo Fisher Scientific) and Mic instrument (bio molecular systems).

13.2. Limit of detection, LoD95%

Method: The limit of detection (LoD95% = smallest number of copies of target DNA which can be detected in 95% of cases) was determined with dilutions of a reference material (AMPLIRUN® Bordetella parapertussis DNA control, order number MBC007, Vircell and AMPLIRUN® Bordetella pertussis DNA control, order number MBC008, Vircell) around the analytical sensitivity using a non-linear curve fitting with the Graph Pad Prism Software.

Result: The LoD95% is 0.06 genome copies/reaction for *B. pertussis* and 0.18 genome copies/reaction for *B. parapertussis*.
The IS481 is a multicopy gene found up to 249 times in the genome of *B. pertussis.* The IS1001 is also multicopy gene, found up to 22 times in the genome of *B. parapertussis*.

13.3. Linearity and dynamic measuring range

Method: Linearity was determined using a 10-fold dilution series of two plasmids containing a fragment of IS418 of *B. pertussis* (10^6 – 10^1 target copies/reaction) and IS1001 of *B. parapertussis* (10^5 – 10^2 target copies/reaction) DNA. The number of determinations (n) per dilution was ten.

Result: For the IS481 gene, the assay shows linearity over the range of 100 to 1,000,000 target copies/reaction with a slope of -4.001 ± 0.04524 and a correlation coefficient R^2 of > 0.99 as shown in Figure 3.
For the IS1001 gene, the assay shows linearity over the range of 100 to 1,000,000 target copies/reaction with a slope of -3.732 ± 0.02664 and an R^2 of > 0.99 as shown in Figure 4.

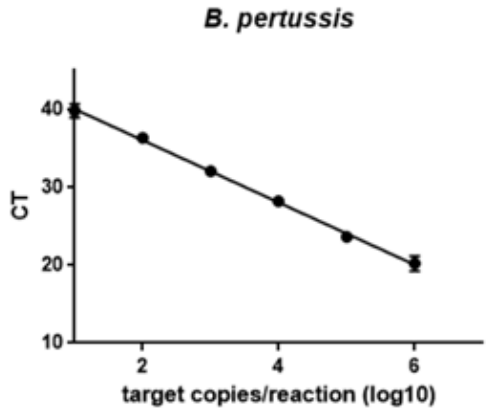


Figure 3 Ten-fold dilution series of a *B. pertussis* DNA standard

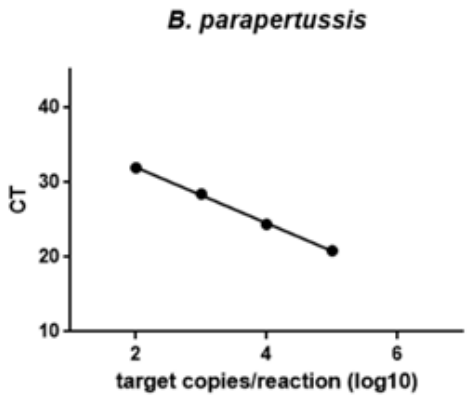


Figure 4 Ten-fold dilution series of a *B. parapertussis* DNA standard

13.4. Precision

Method: Precision within a run (intra-assay), between multiple runs (inter-assay) and between two lots (inter-lot) was determined.

Result: For the IS481 target, the mean values of the coefficients of variation (CV%) are 0.73% for intra-assay precision, 0.7% for inter-assay precision and 1.9% for inter-lot precision.
For the IS1001 target, the mean values of the coefficients of variation (CV%) are 0.7% for intra-assay precision, 0.3% for inter-assay precision and 0.15% for inter-lot precision.

13.5. Analytical specificity

Method BLAST analysis: The selection of highly specific primers and probes ensures analytical specificity. The specificity of primer and probes was validated *in silico* using the Basic Local Alignment Search Tool (BLAST) against the NCBI database. Primers and probes have been checked for possible homologies to currently published sequences. This analysis validates the detection of so far known strains of *Bordetella pertussis, Bordetella parapertussis, Bordetella holmesii* and *Bordetella bronchiseptica*.

Result: No relevant sequence variabilities were observed in the primer and probe region of *Bordetella pertussis, Bordetella parapertussis, Bordetella holmesii* and *Bordetella bronchiseptica* strains. Analysis shows a cross-reaction with *Achromobacter denitrificans* (VIC channel).

Method testing exclusivity: Analytical specificity has been further evaluated by testing genomic DNA of *Borrelia afzelii, Mycoplasma pneumoniae, Streptococcus pneumoniae, Staphylococcus aureus, Haemophilus influenzae, Chlamydophila pneumoniae, Neisseria meningitidis, Escherichia coli*.

Result: No cross-reactions have been observed.

13.6. Diagnostic evaluation

Method:
The diagnostic evaluation was performed by an external service provider using 181 DNA isolates from clinical samples that had been tested in advance using a DIN EN ISO 15189 accredited reference method. Samples included sputum (n=7), BAL (n=39), swabs (nasal, throat; n=101), bronchial secretion (n=18), tracheal secretion (n=15) and pleural punctate (n=1). DNA was isolated from clinical specimens using the Qiagen QiaAmp Mini Kit (n=57), Roche High Pure PCR Template Preparation Kit (n=44), Roche MagNA Pure Compact (n=3) as well as the Roche MagNAPure 96 System (n=77; MagNA Pure 96 DNA and Viral NA Small Volume Kit; Universal Pathogen 200 protocol; 200 µl sample input).
Samples represented 81 samples positively tested for *Bordetella* and 100 *Bordetella*-negative samples. The 81 *Bordetella* positive samples included the following species: *B. pertussis* (n=58), *B. parapertussis* (n=15), *B. pertussis+B. parapertussis* (n=1), *B. bronchiseptica* (n=3), *B. holmesii* (n=4).

The reference method was a DIN EN ISO 15189 accredited pathogen-specific quantitative real-time PCR reference method detecting the insertion sequence IS481 of *B. pertussis, B. holmesii* and *B. bronchiseptica* as well as the insertion sequence IS1001 of *B. parapertussis*, *B. bronchiseptica* and *B. holmesii* using hybridization probes (Kösters et al. 2002). For each sample, the human beta-globin gene was additionally detected as an extraction and inhibition control using an in-house developed, accredited real-time PCR method. Real-time PCR was performed using a LightCycler® 480 II (Roche Diagnostics).

Result:
All 81 samples which were positive for *Bordetella* with the reference method were also positive with BactoReal® Kit *Bordetella* Multiplex (Cq values between 13-40). The remaining 100 DNA samples which were negative with the reference method were also negative with BactoReal® Kit *Bordetella* Multiplex.

The diagnostic evaluation of BactoReal® Kit *Bordetella* Multiplex is shown in Table 4 and Table 5.

Regarding the correct identification of the *Bordetella* species, see Table 6.

Table 4 Overall results obtained with 181 tested clinical isolates, 2x2 contingency table

	Reference			
		pos	**neg**	**Total**
BactoReal® Kit *Bordetella* Multiplex	**pos**	**81**	**0**	**81**
	neg	**0**	**100**	**100**
	Total	**81**	**100**	**181**

Table 5 Statistical evaluation of the diagnostic validation

	Value	95% CI
Sensitivity	100.00%	95.55% to 100.00%
Specificity	100.00%	96.38% to 100.00%
NPV	100.00%	
PPV	100.00%	
Prevalence	44.75%	37.37% to 52.31%

Table 6 Species identification

Species identification	
Reference method	**BactoReal® Kit *Bordetella* Multiplex**
B. pertussis (n=58)	*B. pertussis/B. bronchiseptica* (n=57) *B. pertussis/B. bronchiseptica* + *B. parapertussi* (Cq=42) (n=1)
B. parapertussis (n=15)	*B. parapertussis/B. bronchiseptica* (n=15)
B. pertussis + *B. parapertussis* (n=1)	*B. pertussis/B. bronchiseptica* + *B. parapertussis* (n=1)
B. bronchiseptica (n=3)	*B. parapertussis/B. bronchiseptica* (n=3)
B. holmesii (n=4)	*B. holmesii* (n=4)

14. References

- Kösters, K., Reischl, U. et al., 2002, JCM, Vol. 40, No. 5, p. 1719–1722.
- Van der Zee A, Agterberg C, van Agterveld M, Peeters M, Mooi FR. 1993. Characterization of IS1001, an insertion sequence element of *Bordetella parapertussis*. J Bacteriol. 175:141-147.
- Register KB, Sanden GN. 2006. Prevalence and sequence variants of IS481 in *Bordetella bronchiseptica*: implications for IS481-based detection of *Bordetella pertussis*. J. Clin. Microbiol. 44:4577-4583.
- Weigand MR, Peng Y, Batra D, Burroughs M, Davis JK, Knipe K, Loparev VN, Johnson T, Juieng P, Rowe LA, Sheth M, Tang K, Unoarumhi Y, Williams MM, Tondella ML. 2019. Conserved Patterns of Symmetric Inversion in the Genome Evolution of *Bordetella* Respiratory Pathogens. mSystems. 4(6):e00702-19.

15. Revision history

Revision	Date	Description
1.0	25 May 2022	First version
1.1	01 May 2023	**Change of the company address** **5. Contents, stability and storage** Kit version 1.1: Filling volume of IPC-Target (DNA) and Positive Control was changed. The DNA IPC Target is not stored in RNA/DNA Stabilizer anymore and was renamed to IPC-Target (DNA). **7. Precautions and safety instructions** Update of this section Inclusion of additional information on specimen collection and storage, processing of specimens after collection, storage conditions of specimens, transport and storage conditions of purified DNA. Recommended measures to avoid DNA contamination added. **9. Preparation of samples** Update of paragraph of Quality control for DNA extraction and PCR inhibition Note that for Cq values < 27, the IPC-Target (DNA) must be diluted prior to extraction. **11. Interpretation of PCR data** Table 1 and Table 2: Update

1.2	24.10.2023	**1. Intended purpose** Revision with respect to *B bronchiseptica* – removal in the listing of identification. **2. Product description** Revision with respect to *B bronchiseptica* – removal in the listing of identification. Inclusion of a further sentence: "Sometimes, a few copies of IS481 or IS1001 may be present in *B. bronchiseptica*, which could possibly be detected if the concentration of bacteria is high." **10.2 Programming of temperature profile** Select detection channels: *B. bronchiseptica* placed in brackets. **11.1 Controls** IS-sequences added to the header of the table. **11.2 Clinical samples** Pathogen replaced with IS sequence in the header of the table, removal of *B. bronchiseptica* from the table, shown as a separate clinical sample.
1.3	29.11.2023	**2. Product description** Addition of CFX Opus Real-time PCR System (Bio-Rad), CFX Dx (Bio-Rad) and T-COR 8 (Tetracore)

Note:
Any serious incident that has occurred in relation to the product shall be reported to the manufacturer and the competent authority of the Member State in which the user and/or the patient is established.

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