# BactoReal® Kit
# *Clostridium difficile*

## Manual

**For use with the**

- **ABI PRISM® 7500 (Fast)**
- **Mx3005P®**
- **LightCycler® 480**


**For veterinary use only**

REF **DVEB04213** Σ **100**

REF **DVEB04253** Σ **50**

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## 1. Product description

BactoReal® Kit *Clostridium difficile* is a real-time PCR assay for detection of DNA of *Clostridium difficile* toxin A and B. This test was developed and validated for the ABI PRISM® 7500 (Fast) instrument (Life Technologies), LightCycler® 480 (Roche) and Mx3005P® (Agilent), but is also suitable for other real-time PCR instruments. This test allows the rapid and sensitive detection of DNA of toxigenic *Clostridium difficile* from samples purified from stool or other sample material (e.g. with the QIAamp DNA Stool Mini Kit).

BactoReal® Kit *Clostridium difficile* allows the rapid and sensitive detection of *Clostridium difficile*. This test is performed in a multiplex real-time PCR format to enable differentiation between *Clostridium difficile* toxin A from toxin B. In VIC/HEX channel the toxin A (tcdA) gene of *Clostridium difficile* is detected. In FAM channel the toxin B (tcdB) gene of *Clostridium difficile* is detected.
An internal positive control system for detection in Cy5 channel (667 nm) excludes false-negative interpretation of results due to inhibition of real-time PCR (see 8. Interpretation of PCR-data).
When using PCR-platforms not validated by ingenetix, an evaluation of the multiplex-PCR is recommended. Please be aware that some PCR-platforms have to be calibrated with the corresponding dye before performing multiplex-PCR.

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

## 2. Pathogen information

*Clostridium difficile* is a gram-positive spore-forming anaerobic bacterium which is commonly found in low concentrations in the intestine of clinically normal animals and humans. In higher concentration *Clostridium difficile* is an important cause of diarrhea in neonatal swine. Affected piglets may have dyspnea, abdominal distention, and scrotal edema. Diarrhea may not be present in all pigs affected. Pathogenic *C. difficile* strains produce multiple toxins. The best characterized are enterotoxin (*Clostridium difficile* toxin A causing fluid secretion into the gut lumen) and cytotoxin (*Clostridium difficile* toxin B), both of which may produce diarrhea and inflammation. *Clostridium difficile* infection (CDI) is also a major cause of nosocomial antibiotic-associated infectious diarrhea and pseudomembranous colitis in humans.

**References:**
Voth DE, Ballard JD. 2005. *Clostridium difficile* toxins: mechanism of action and role in disease. Clin. Microbiol. Rev.18:247-63.

## 3. Principle of real-time PCR

A specific DNA sequence of the pathogen genome is amplified and the generated PCR-product is detected by an oligonucleotide-probe labelled with a fluorescent dye. This technology allows for a sequence-specific detection of PCR amplificates.

## 4. General Precautions

The user should always pay attention to the following:
- Always include a negative control per PCR-run (water instead of sample).
- Optional: for valid interpretation of results, a negative control should be included during DNA-extraction (for example extraction of water instead of sample material), in order to exclude false-positive results due to contamination with *Clostridium difficile* DNA during extraction.
- Be careful when handling the positive control.
- Store and extract positive material (specimens, controls and amplicons) separately from all other reagents and add it to the reaction mix in a spatially separated workspace.
- Periodically decontaminate benches and devices.
- Use sterile pipette tips with filters.
- Thaw all components thoroughly at room temperature before starting an assay. When thawed, mix the components and centrifuge briefly.
- For MSDS, see www.ingenetix.com.

## 5. Contents of the Kit

| Labelling | Content | Amount | | Storage |
|---|---|---|---|---|
| | | DVEB04213 | DVEB04253 | |
| *C. difficile* tcdA&B Assay Mix (green cap) | Primer and probe (VIC/HEX+FAM) for detection of toxin A&B of *C. difficile* | 2 x 50 µl | 1 x 50 µl | -20°C |
| CR-3 Assay Mix (yellow cap) | Primer, probe (Cy5) and target for detection of IPC | 2 x 50 µl | 1 x 50 µl | -20°C |
| *C. difficile* tcdA&B Positive Control (red cap) | Control-DNA (approx. 10,000 target copies/µl) | 1 x 25 µl | 1 x 25 µl | -20°C |
| DNA Reaction Mix (white cap)[#] | Reaction Mix | 2 x 500 µl | 1 x 500 µl | **-20°C until first use, then at +4°C** |
| Water (blue cap) | Water | 1 x 1000 µl | 1 x 1000 µl | -20°C to +4°C |

[#]DNA Reaction Mix contains uracil-N glycosylase (UNG)

The components of BactoReal® Kit *Clostridium difficile* are stable until the expiry date stated on the label. Repeated thawing and freezing should be avoided. Please protect kit components from light.

## 6. Additionally required materials and devices

- Reagents and devices for DNA-extraction
- PCR-grade water
- Disposable powder-free gloves
- Pipettes (adjustable)
- Sterile pipette tips with filters
- Vortex mixer
- Desktop centrifuge with rotor for 2 ml reaction tubes
- Real-time PCR instrument which is able to detect and differentiate fluorescence in FAM, VIC/HEX and Cy5 channel
- Appropriate 96 well reaction plates or reaction tubes with corresponding (optical) closing material

## 7. Preparation of real-time PCR

Please make sure that at least one negative control (water, blue cap), as well as one positive control (red cap) and one extraction negative control (optional, recommended) are included per PCR run.
Ingenetix highly recommends performing PCR analyses in duplicates, which increases the probability of detection of the pathogen and facilitates interpretation of results.

### 7.1. Pipetting scheme

| | | Per sample |
|---|---|---|
| **Preparation of Master Mix** (mix well) | Water* | 3.0 µl |
| | DNA Reaction Mix (2x) | 10.0 µl |
| | *C. difficile* tcdA&B Assay Mix | 1.0 µl |
| | CR-3 Assay Mix | 1.0 µl |
| | **Total volume Master Mix** | **15.0 µl** |
| **Preparation of PCR** | Master Mix | 15.0 µl |
| | Sample* | 5.0 µl |
| | **Total volume** | **20.0 µl** |

*1-8 µl of the sample can be used. When using an amount other than 5 µl of the sample, the amount of $H_2O$ has to be changed accordingly.

**Positive Control:** As positive control use 1 µl of the *C. difficile* tcdA&B Positive Control + 4 µl $H_2O$.
Optional: a 1:10 dilution of the positive control can be used and defined as second standard value (approx. 1000 target copies/µl).

### 7.2. Programming of the temperature profile

Please find further information on programming the real-time PCR instrument in the respective operator's manual. Please be aware that some PCR-platforms have to be calibrated with the corresponding dye before performing multiplex-PCR.

**Select dyes:** VIC/HEX-TAMRA for detection of toxin A of *C. difficile*
FAM-TAMRA for detection of toxin B of *C. difficile*
Cy5-NONE (CR-3 Assay Mix) for detection of IPC
**Select reference dye (passive reference)**: ROX
**Sample Volume:** 20 µl
**Temperature Profile:**

| Program 1 | Program 2 | Program 3 |
|---|---|---|
| Cycles: 1<br>Analysis: None | Cycles: 1<br>Analysis: None | Cycles: 45<br>Analysis: Quantification<br>Acquisition at 60° |
| 50°C<br>2 min* | 95°C<br>20 sec | 95°C<br>5 sec<br>60°C<br>1 min |

<u>For ABI PRISM® 7500:</u>
Ramp speed: Without "fast cycling" parameter

<u>For LightCycler® 480 instrument:</u>
Detection format: 3 Color Hydrolysis Probe (dyes see above)

***Note:** If viral RNA should be also detected in the same PCR run, program 1 has to be prolonged to 15 min at 50°C. This temperature profile can be used for all BactoReal®, MycoReal, ParoReal and ViroReal® kits for the detection of DNA or RNA.

## 8. Interpretation of PCR-data

Examples for interpretation of positive reactions are shown in the amplification plots below.

**For a valid interpretation, the following criteria must be fulfilled:**

| | Ct/Cp (VIC/HEX channel) Toxin A target | Ct/Cp (FAM channel) Toxin B target | Ct/Cp (Cy5 channel) IPC target | Interpretation |
|---|---|---|---|---|
| Negative control | Negative | Negative | 36.0 ± 2 | Valid |
| Positive control (undiluted, 1 µl/PCR) | 27.0-30.0 | 27.0-30.0 | 36.0 ± 2 | Valid |
| Or: positive control (1:10 diluted, 1 µl) | 30.0-33.0 | 30.0-33.0 | 36.0 ± 2 | Valid |
| Extraction negative control (optional) | Negative | Negative | 36.0 ± 2 | Valid |
| Negative sample | Negative | Negative | 36.0 ± 2 | Valid |
| Sample: *C. difficile* **negative** | **Negative** | **Negative** | Positive/Negative | Valid |
| Sample: *C. difficile* **positive** | **Positive** | **Positive** | Positive/Negative | Valid |
| Sample: *C. difficile* **positive** | **Negative** | **Positive** | Positive/Negative | Valid |
| Sample: *C. difficile* **positive** | **Positive** | **Negative** | Positive/Negative | Valid |

**For analysis of PCR data please proceed as follows:**
For analysis of PCR results gained with BactoReal® Kit *Clostridium difficile* please select fluorescence display options VIC/HEX and FAM channel for the *Clostridium difficile* toxin A and B target and Cy5 channel for the internal positive control target. Samples with a positive Cp or Ct-value in VIC/HEX and/or FAM channel are considered positive for *Clostridium difficile*. Please also check the presence of amplification-curves manually.

**Once the analysis is completed, the following results are possible:**

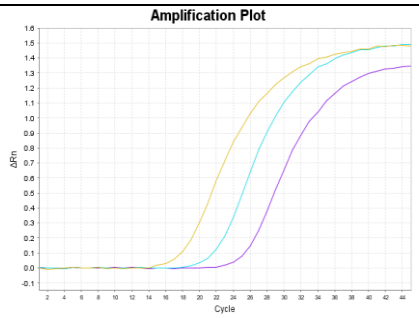
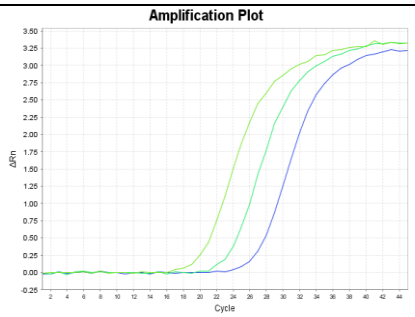
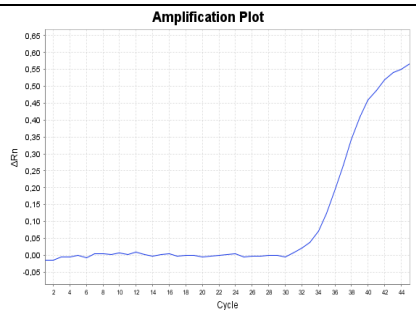
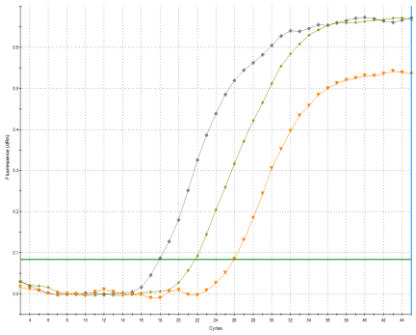
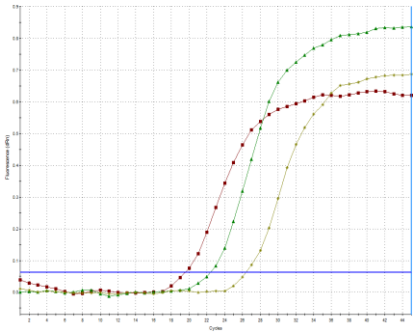
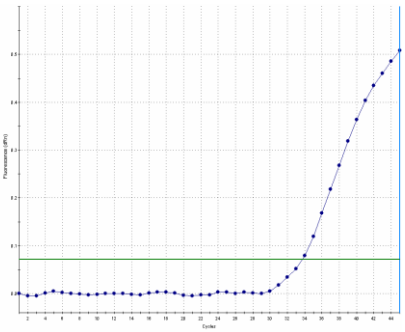
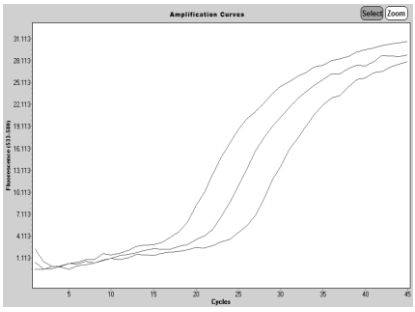
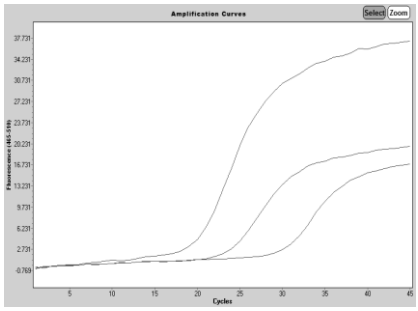
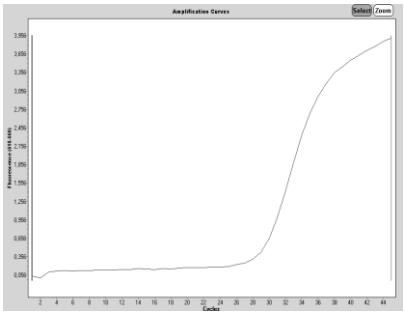
**1. Signal in VIC/HEX and FAM channel:**
→ DNA of *Clostridium difficile* toxin A and B was amplified. The sample has to be interpreted as positive.
*Clostridium difficile* DNA can lead to a reduced or absent fluorescence signal of the internal positive control (competition of PCR).

**2. Signal in VIC/HEX channel but no signal in FAM channel:**
→ DNA of *Clostridium difficile* toxin A was amplified. The sample has to be interpreted as positive.
*Clostridium difficile* DNA can lead to a reduced or absent fluorescence signal of the internal positive control (competition of PCR).

**3. Signal in FAM channel but no signal in VIC/HEX channel:**
DNA of *Clostridium difficile* toxin B was amplified. The sample has to be interpreted as positive.
*Clostridium difficile* DNA can lead to a reduced or absent fluorescence signal of the internal positive control (competition of PCR).

**4. No signal in VIC/HEX and FAM channel but signal of the internal positive control in Cy5 channel:**
→ No *Clostridium difficile* DNA is detectable in the sample. The sample has to be interpreted as negative.
The positive signal of the internal positive control assay excludes a putative PCR inhibition.

**5. No signals in VIC/HEX and FAM channel and no signal with internal positive control in Cy5 channel:**
→ No interpretation statement can be made.
Information about possible sources of error and their solution can be found in 9. Troubleshooting.

| Detection of ***Clostridium difficile* toxin A** | Detection of ***Clostridium difficile* toxin B** | Detection of internal positive control **CR-3** |
|---|---|---|
|  |  |  |
| **ABI Prism® 7500:** VIC channel, 554 nm<br>1:10 serial dilution of *C. difficile* DNA (tcdA) | **ABI Prism® 7500:** FAM channel, 530 nm<br>1:10 serial dilution of *C. difficile* DNA (tcdB) | **ABI Prism® 7500:** Cy5 channel, 667 nm<br>Internal positive control |
|  |  |  |
| **Mx3005P®:** HEX channel<br>1:10 serial dilution of *C. difficile* DNA (tcdA) | **Mx3005P®:** FAM channel<br>1:10 serial dilution of *C. difficile* DNA (tcdB) | **Mx3005P®:** CY5 channel<br>Internal positive control |
|  |  |  |
| **LightCycler® 480:** VIC/HEX channel<br>1:10 serial dilution of *C. difficile* DNA (tcdA) | **LightCycler® 480:** FAM channel<br>1:10 serial dilution of *C. difficile* DNA (tcdB) | **LightCycler® 480:** Cy5 channel, 667 nm<br>Internal positive control |

## 9. Troubleshooting

**1. No *C. difficile* specific signal with positive control:**

- Incorrect programming of the temperature profile of the real-time PCR instrument.
  → Compare the temperature profile with the protocol (see 7. Preparation of real-time PCR).
- Incorrect configuration of the PCR reaction.
  → Check your work steps (see 7. Preparation of real-time PCR) and repeat the PCR, if necessary.

**2. No signal with the internal positive control and no *C. difficile* specific signal with the sample:**

- The PCR reaction was inhibited. No interpretation can be made.
  → Make sure that you use a recommended method for DNA isolation and stick closely to the manufacturer's instructions.
  → If no operating mistakes during 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 amount of $H_2O$).
- Incorrect PCR conditions.
  → Check the PCR conditions and repeat the PCR, if necessary.

**3. *Clostridium difficile* specific signal with the negative control:**

- A contamination occurred during preparation of the PCR.
  → Repeat the PCR with new reagents in replicates.
  → Strictly pipette the positive controls at last.
  → Make sure that work space and instruments are decontaminated at regular intervals.

**4. *Clostridium difficile* specific signal with the negative control of DNA-extraction:**

- A contamination occurred during extraction.
  → Repeat the extraction and PCR using new reagents.
  → Make sure that work space and instruments are decontaminated at regular intervals.

## 10. Specifications

BactoReal® Kit *Clostridium difficile* was evaluated with the ABI PRISM® 7500 (Fast) instrument (Life Technologies), with the LightCycler® 480 (Roche) and the Mx3005P® (Agilent). For further validation data please contact ingenetix.

### 10.1. Analytical sensitivity

The analytical sensitivity for toxA is 20 target copies/reaction and for toxB 10 target copies/reaction. The limit of detection (LoD95 = smallest number of copies of target DNA which can be detected in 95% of cases) of 52 target copies/reaction for toxA and of 33 target copies/reaction for toxB was determined by several replicates around the detection limit.

### 10.2. Analytical specificity

The specificity is ensured by the selection of highly specific primers and probes. The primers and probes were checked for possible homologies to currently published sequences by sequence comparison analyses. This also validated the detection of so far known *Clostridium difficile* strains carrying the toxin A and/or toxin B.

## 11. Annex – symbols

| | | | |
|---|---|---|---|
| LOT | Batch code | | Use by |
| REF | Catalogue number | | Manufactured by |
| Σ | Contains sufficient for <n> tests | | Store at |