# BactoReal® Kit *E. coli & Klebsiella*

## Kit version 1.1

# Instructions for Use

CE

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

REF **DHUS00153**

Σ **50 reactions**

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The MGB Probe contained in this product is covered by one or more of the following US patents and corresponding patents outside the US: 5801155 and 6084102 and is sold under a license from the ELITech Group. The purchase of this product includes a license to use only this amount of product solely for the purchaser´s own use solely in the human in vitro diagnostic field (in accordance with applicable FDA and other regulatory requirements) and may not be used for any other commercial use, including without limitation repacking or resale in any form.

## 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 *E. coli* & *Klebsiella* is a non-automated IVD real-time PCR test for the qualitative detection and identification of DNA of *Escherichia coli* (dxs gene), *Klebsiella pneumoniae, K. quasipneumoniae, K. variicola, K. oxytoca* and *K. aerogenes* (khe gene).
Proper specimens are DNA extracts isolated from samples of human EDTA blood, aspirates, CSF and biopsies.
In combination with other diagnostic tests such as culture the test supports a rapid and specific diagnosis for patients suspected of having a bacterial infection. Results must be interpreted in context of the overall picture and other clinical parameters. The diagnosis of sepsis must not be based solely on BactoReal® Kit *E. coli* & *Klebsiella*.

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 *E. coli* & *Klebsiella* is a real-time PCR test and detects the dxs gene of *Escherichia coli* and the khe gene of *Klebsiella pneumoniae, K. quasipneumoniae, K. variicola, K. oxytoca* and *K. aerogenes.*

Probe-specific amplification curves in the fluorescence channel for FAM and VIC detect *E. coli* and *Klebsiella.* 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 with the cobas z 480 Analyzer (Roche). 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, QuantStudio™ 7 real-time PCR system (Thermo Fisher Scientific), qTOWER$^3$G (Analytik Jena), Mic instrument (bio molecular systems), LightCycler® 480 II (Roche)).

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.

Further ingenetix tests for diagnosis of sepsis:
BactoReal® Kit *Staphylococcus* spp. & *S. aureus*, order no.: DHUS00253
BactoReal® Kit *S. pneumoniae* & *Streptococcus* spp., order no.: DHUS00353
BactoReal® Kit vanA & *Enterococcus* spp., order no.: DHUS00453
BactoReal® Kit *P. aeruginosa* & *Enterobacter* spp., order no.: DHUS00553
BactoReal® Kit *A. baumannii* & *P. mirabilis*, order no.: DHUS00653
MycoReal® Kit *Candida* & *A. fumigatus*, order no.: DHUF00153

Ingenetix BactoReal®, ViroReal®, MycoReal®, PanReal und ParoReal® Kits sind darauf optimiert, mit dem gleichen Temperaturprofil den Nachweis von DNA und RNA in einem PCR Lauf zu ermöglichen.

# 3. Pathogen information

*Escherichia coli* is a gram-negative bacterium inhabiting the normal intestinal flora. Most *E. coli* strains are non-pathogenic but some strains of this species can cause disease such as urinary tract infections, gastrointestinal infections, or sepsis.
*Klebsiella* species are gram-negative, facultatively anaerobic bacteria found ubiquitously in the environment. They are part of the healthy gut flora in humans. Outside the intestine they can lead to infections such as pneumonia and sepsis. Some strains show antibiotic resistance and are therefore difficult to treat. Both *E. coli* and *Klebsiella* are microorganisms responsible for nosocomial infections.

## 4. Principle of real-time PCR

The test is based on multiplex real-time PCR technology 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** |
|---|---|---|---|
| Module 1 + IPC3 Assay Mix (green cap) | Primers and probes for detection of<br>- *E. coli* (FAM)<br>- *Klebsiella* (VIC)<br>- 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 |
| Module 1 Positive Control (red cap) | DNA positive control (approx. 1,000 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 dry ice or with coolpacks. 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.
- Protect kit components from light.
- The real-time PCR instrument should be calibrated, serviced, and cleaned regularly.
- 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.
- This kit has been used to validate the detection of bacterial DNA from EDTA blood, aspirates, CSF, and biopsies. If the concentration of leukocytes in the blood is low, pathogen DNA may be lost during extraction.
- A negative test result does not exclude the possibility of infection with pathogenic organisms.
- Sequence variabilities in the target-region of some subtypes (strains) may lead to false-negative or less sensitive results.
- A pathogen concentration in the sample below the detection limit can lead to a false-negative result.
- A cut-off must be established for *E. coli* to avoid false-positive data due to contamination, which reduces the sensitivity of the test.
- Sepsis diagnosis must not be based solely on BactoReal® Kit *E. coli* & *Klebsiella*. The test has to be used in combination with other diagnostic tests such as culture to enable a rapid and specific diagnosis for patients suspected of having sepsis or a bacterial infection of the bloodstream.
- Results should be interpreted in context of other clinical and laboratory findings.

# 9. Preparation of samples

BactoReal® Kit *E. coli* & *Klebsiella* is suitable for analysis of DNA extracted from human EDTA blood, aspirates, CSF, and biopsies.

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

Aspirates and cerebrospinal fluid can be stored in microcentrifuge tubes. 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.

Biopsies can be stored in microcentrifuge tubes containing sterile physiological saline (approx. 100 µl) at 2-8 °C for up to 48 h. If it is not guaranteed that the sample will be processed within 48 h, the sample shall be immediately frozen dry without additives at -20/-80 °C.

Blood has to be stored in EDTA blood collection tubes. 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 *E. coli* & *Klebsiella*. Extract the sample using a DNA extraction system compatible with real-time PCR technology and appropriate for the sample material.

For manual extraction recommended
- QIAamp DNA Mini Kit (Qiagen)

For automated extraction recommended
- innuPREP AniPath DNA RNA – KFFLX Kit (Analytik Jena) with the KingFisher FLEX instrument (Thermo Fisher Scientific)

### 9.2.1. Extraction of EDTA blood

The extracted DNA analyzed per module should be equivalent to 50 µl of blood at least (e.g., 500 µl of blood eluted in 100 µl). If the sample is planned to be further screened for other sepsis pathogens, extraction of 1 ml EDTA blood in combination with mechanical lysis by beads is recommended.

For automated extraction of EDTA blood recommended
- Optional: mechanical lysis of 1.3 ml EDTA blood with beads (e.g., MP Biomedicals™ Lysing Matrix E, 2 ml, mpbio) on the Magnalyser or another beadbeater (70 sec, 7000 rpm).
- Subsequently, automated extraction of supernatant (approx. 800 µl) with the innuPREP AniPath DNA RNA - KFFLX Kit (Analytik Jena) with a modified protocol for 800 µl sample volume using the KingFisher FLEX extraction device (Thermo Fisher Scientific).

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 1 µl IPC-Target (DNA) (orange cap) directly into the appropriate volume of lysis buffer (or spike it to the sample after 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

- The preparation of the real-time PCR should be done in a PCR workstation (see point 7.2).
- 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 and module |
|---|---|---|
| **Preparation Master Mix per module** (mix well) | DNA Reaction Mix | 10.0 µl |
| | Module 1 + IPC3 Assay Mix | 1.0 µl |
| | **Module Master Mix** | **11.0 µl** |
| **PCR-reaction** | Module 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 Instrument, QuantStudio™ (Thermo Fisher Scientific)

| **Program 1**<br>Cycles: 1<br>Analysis: None | **Program 2**<br>Cycles: 1<br>Analysis: None | **Program 3**<br>Cycles: 45<br>Analysis: Quantification<br>Acquisition at 60 °C |
|---|---|---|
| 50 °C<br>2 min | 95 °C<br>2 min | 95 °C<br>5 sec<br>60 °C<br>30 sec |

**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 ViroReal®, BactoReal®, 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.
**Ad program 3:** 1 minute possible in combination with other ingenetix kits.

**<u>Select detection channels</u>**

**FAM channel:** Detection of *E. coli*
**VIC channel:** Detection of *Klebsiella*
**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 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:**
Low concentrations of pathogen DNA can often be present in the PCR and extraction reagents. Therefore, Cq values of the samples in the FAM and VIC channel must be interpreted in comparison to Cq values of the negative control of DNA extraction also. Defined cut-off Cq values apply to the pathogens listed below.

Cut-off Cq-values:
Contamination with *E. coli* is possible during sampling, extraction and PCR. Therefore, a cut-off with Cq=35 must be defined for *E. coli*. The sensitivity of the test (limit of detection, LoD) is reduced accordingly, see Table 4.

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 was added during extraction.

| | Cq FAM channel *E. coli* | Cq VIC channel *Klebsiella* | Cq Cy5 channel IPC[1] | Interpretation | Action |
|---|---|---|---|---|---|
| **Positive control** | < 30 | < 30 | Negative | Valid | - |
| **Positive control** | Negative | Negative | Negative | Invalid | See 12.1 |
| **Positive control** | < 30 | Negative | Negative | Invalid | See 12.1 |
| **Positive control** | Negative | < 30 | Negative | Invalid | See 12.1 |
| **Extraction negative control[3]** | > 35 | Negative | 27-30[2] | Valid | - |
| **Extraction negative control[3]** | > 35 | Negative | Negative | Invalid | See 12.1 |
| **Extraction negative control[3]** | < 35 | Positive | 27-30[2] | Invalid | See 12.3 |
| **Extraction negative control[3]** | < 35 | Negative | 27-30[2] | Invalid | See 12.3 |
| **Extraction negative control[3]** | > 35 | Positive | 27-30[2] | Invalid | See 12.3 |
| **NTC PCR (optional)** | > 35 | Negative | Negative | Valid | - |
| **NTC PCR (optional)** | < 35 | Positive | Negative | Invalid | See 12.2 |
| **NTC PCR (optional)** | > 35 | Positive | Negative | Invalid | See 12.2 |
| **NTC PCR (optional)** | < 35 | Negative | Negative | Invalid | See 12.2 |

[1] If the IPC Target has been added directly to the Master Mix, all samples must be positive in Cy5 channel
[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).
[3] Negative control of the extraction. Cut-off for *E. coli*: Cq=35.0

### 11.2. Clinical samples

The results of the negative control of extraction as well as the cut-off must be included in the interpretation, see Table 3.

**FAM channel, *E. coli*:** For *E. coli* a Cq-value cut-off must be defined. The Cq value for the cut-off is 35.0 (corresponds to approx. 50 target copies). Samples with Cq values lower than 35.0 are considered positive results, while Cq values greater than 35.0 may represent contamination and should be considered negative results.
**VIC channel, *Klebsiella*:** Samples with positive Cq values are interpreted as positive.

**Table 3** Interpretation of clinical samples

| | Cq FAM channel *E. coli* | Cq VIC channel *Klebsiella* | Cq Cy5 channel DNA IPC Target | Interpretation (see also Table 6) | Action |
|---|---|---|---|---|---|
| **Clinical sample** | > 35 | Negative | 27-30[1] | Negativ | - |
| **Clinical sample** | < 35 | Positive | Positive/Negative[2] | Positive for *E. coli* + *Klebsiella* | - |
| **Clinical sample** | < 35 | Negative | Positive/Negative[2] | Positive for *E. coli* | - |
| **Clinical sample** | > 35 | Positive | Positive/Negative[2] | Positive for *Klebsiella* | - |
| **Clinical sample** | > 35 | Negative | Negative | Invalid | See 12.5 |

[1] A positive signal of the DNA 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.

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 DNA IPC Target 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 DNA IPC Target must be added to the master mix. If the DNA IPC Target has been forgotten to be added:
  → Freshly dilute DNA IPC Target and repeat PCR.
- For control of DNA extraction and PCR inhibition, the undiluted DNA IPC Target 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 DNA IPC Target has been added to lysis buffer during extraction, but there is IPC specific signal with PCR-NTC and positive control: Contamination with the DNA IPC Target.
  → 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 DNA IPC Target was added during extraction:
  - Inhibition of PCR may have occurred.
  - DNA extraction was unsuccessful.
  - The DNA IPC target was not added to the lysis buffer of the sample.
  - The extracted sample was not added to the PCR-reaction.

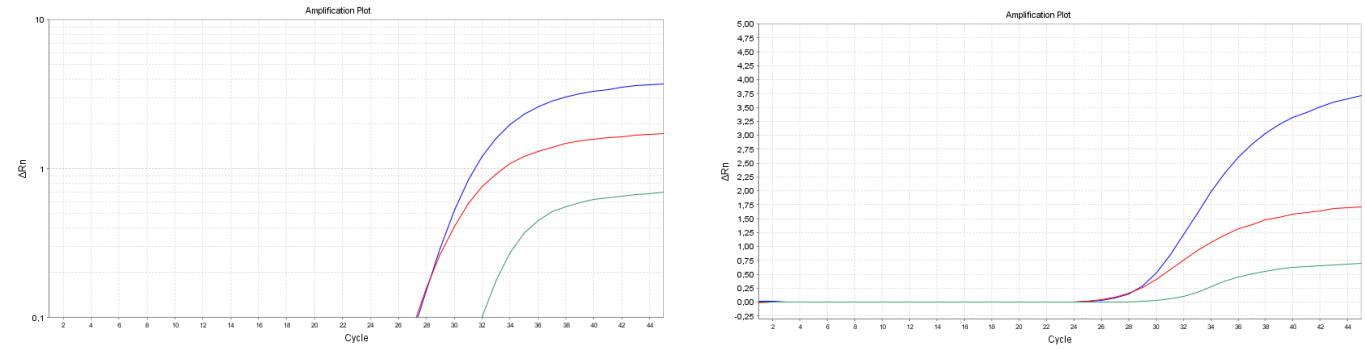
  → No statement is possible. Verify you use a recommended method for DNA isolation and re-examine the single steps of the DNA extraction.
  → If no operating mistakes during DNA extractions can be retraced, it is recommended to repeat the PCR with lower amounts of DNA-eluate (1/5 or 1/10 of sample volume + 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 *E. coli* & *Klebsiella* with an ABI® 7500 instrument (Thermo Fisher Scientific) is shown in Figure 1.

**Figure 1** Performance of BactoReal® Kit *E. coli & Klebsiella*




Positive Control: FAM (*E. coli*), VIC (*Klebsiella*) and CY5 (IPC) channel

This test has been validated with the ABI® 7500 Real-time PCR System (Thermo Fisher Scientific) and with the cobas z 480 Analyzer (Roche).

## 13.2. Limit of detection, LoD95%

**Method:** BactoReal® Kit *E. coli* & *Klebsiella* was tested with dilution series of plasmids containing parts of the respective target DNA. The limit of detection (LoD95% = smallest number of copies of target DNA which can be detected in 95% of cases) was determined with plasmid dilutions around the analytical sensitivity using a non-linear curve fitting with the Graph Pad Prism Software.
Furthermore, two different extraction kits (innuPREP AniPath DNA RNA - KFFLX Kit, Analytik Jena, and MagMAX™ Viral/Pathogen Ultra Nucleic Acid Isolation Kit, Thermo Fisher Scientific) including an additional step with mechanical lysis by beads were tested with 1.3 ml EDTA blood spiked with different amounts of *K. pneumoniae* using the KingFisher FLEX extraction device.

**Results:** The results are shown in Table 4. A cut-off with Cq=35 must be defined for *E. coli*. The sensitivity of the test (limit of detection, LoD95%) is reduced accordingly.
Validation of extraction revealed that at least 100 CFU (colony forming units) of *Klebsiella* per ml blood can be detected with BactoReal® Kit *E. coli* & *Klebsiella.*
Note: Sequence variability in the primer or probe region may lead to false-negative or less sensitive results for some pathogen strains.

**Table 4** Summary of LoD and linearity

| | LoD95%<br>Without / with cut-off rule | Linearity – Slope | Linearity – $R^2$ |
|---|---|---|---|
| ***E. coli*** | 7 / 50 | -3.26 ± 0.03 | 0.9966 |
| ***K. pneumoniae*** | 9 | -3.30 ± 0.02 | 0.9972 |

## 13.3. Linearity and dynamic range

**Method:** Linearity was determined using 10-fold dilution series ($10^6$ – $10^1$ target copies/reaction) of the plasmids. The number of determinations (n) per dilution was nine.

**Results:** Results are shown in Table 4. The test shows linearity over the range of 100 to 1,000,000 target copies/reaction.

## 13.4. Precision

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

**Result:** The mean values of the coefficients of variation (CV%) are summarized in Table 5.

**Table 5** Summary of mean values of the coefficients of variation

| | Coefficients of variation intra-assay | Coefficients of variation inter-assay | Coefficients of variation inter-lot |
|---|---|---|---|
| ***E. coli*** | 0.82 | 0.46 | 0.67 |
| ***K. pneumoniae*** | 0.47 | 0.38 | 2.49 |

## 13.5. Analytical specificity

**Method:** 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 database analysis validated the detection of currently known strains and potential cross-reactions.
Specificity was further determined by testing different bacterial and fungal isolates.

**Result:** The results of the BLAST analyses in terms of detection of different species are summarized in Table 5. Existing sequence variability in the target region of some subtypes (strains) may lead to false-negative or less sensitive results.
When testing different bacterial and fungal isolates, no significant cross-reactions were observed (data not shown).

**Table 6:** Results of BLAST analysis

| | Detected species |
|---|---|
| **FAM channel, *Escherichia coli*** | *Escherichia coli* |
| **VIC channel, *Klebsiella*** | *Klebsiella pneumoniae, Klebsiella quasipneumoniae, Klebsiella variicola, Klebsiella oxytoca, Klebsiella aerogenes* |

## 13.6. Clinical evaluation

**Method:** 25 blood samples (extracted with SeptiFast LysKit, Roche) and 9 other specimens such as biopsies, CSF and aspirates of patients with suspected bacterial infection were analyzed. The results were compared with the LightCycler® SeptiFast Test (Roche) for blood samples and the SepsiTest™ (Molzym) for the other test materials.

**Result:** see Table 7.

**Table 7** Statistical clinical evaluation for blood samples and other samples (biopsies, CSF, aspirates)

| | Sensitivity | | Specificity | | NPV | | PPV | | Prevalence | | Accuracy | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Blood | Other | Blood | Other | Blood | Other | Blood | Other | Blood | Other | Blood | Other |
| ***E. coli*** | 100.0% | 100.0% | 81.3% | 100.0% | 100.0% | 100.0% | 75.0% | 100.0% | 36.0% | 22.2% | 88.0% | 100.0% |
| ***Klebsiella*** | 100.0% | 100.0% | 100.0% | 100.0% | 100.0% | 100.0% | 100.0% | 100.0% | 44.0% | 22.2% | 100.0% | 100.0% |

# 14. Literature

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## 15. Revision history

| Revision | Date | Description |
|---|---|---|
| 1.1en | 01.05.2023 | **Change of the company address**<br><br>**1. Intended use**<br>Intended use was specified in more detail.<br><br>**5. Contents, stability and storage**<br>Kit version 1.1:<br>Filling volume of IPC-Target (DNA) and Positive Control was changed.<br>The DNA IPC Target is not stored in RNA/DNA Stabilizer anymore and was renamed to IPC-Target (DNA).<br>Module 1 Assay Mix was renamed to Module 1 + IPC3 Assay Mix.<br><br>**6. Additionally required materials and equipment**<br>Update<br><br>**7. Precautions and safety instructions**<br>Update of this section<br>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.<br>Recommended measures to avoid DNA contamination added.<br><br>**9. Preparation of samples**<br>Information on sample storage was added.<br>MagMAX™ Viral/Pathogen Ultra Nucleic Acid Isolation Kit has been removed.<br>innuPREP AniPath DNA RNA - KFFLX Kit added<br>The DNA IPC Target is not stored in RNA/DNA Stabilizer anymore.<br>Note that for Cq values < 27, the IPC-Target (DNA) must be diluted prior to extraction.<br><br>**10. preparation of real-time PCR**<br>The temperature profile has changed - the activation step in program 2 has been extended from 20 sec to 2 min.<br><br>**11. Interpretation of PCR data**<br>Definition of Cq, Ct and Cp values.<br>Update of tables for results with positive and negative controls and clinical samples.<br>Inclusion of an explanation of how to evaluate clinical specimen test results in relation to controls.<br><br>**12. Troubleshooting**<br>Update regarding references in the new results tables.<br><br>**13. Specifications and performance evaluation**<br>Update. |

**Note:**
Any serious incident that has occurred in relation to the device 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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