BactoReal® Kit *E. coli* Typing

Manual

For use with the

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

For veterinary use only

BactoReal® Kit *E. coli* Typing stx1 & stx2 (STEC)	REF	DVEB04013	Σ 100	
	REF	DVEB04053	Σ 50	
BactoReal® Kit *E. coli* Typing eae & ehxA (EHEC, EPEC)	REF	DVEB04113	Σ 100	
	REF	DVEB04153	Σ 50	
BactoReal® Kit *E. coli* Typing eae (EHEC or EPEC)	REF	DVEB04313	Σ 100	
	REF	DVEB04353	Σ 50	
BactoReal® Kit *E. coli* Typing ehxA (EHEC)	REF	DVEB04413	Σ 100	
	REF	DVEB04453	Σ 50	

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Explanation of symbols

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

1. Product description

BactoReal® Kit *E. coli* Typing is a series of different real-time PCR kits for detection of virulence-associated genes of *E. coli* (genes stx1, stx2, eae and ehxA). Depending on which kit and which combinations of kits are used, they allow the typing of enterohemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC) and of Shiga toxin (verotoxin) producing *E. coli* (STEC).

Tests were developed and validated for the ABI PRISM® 7500 (Fast) instrument (Life Technologies), LightCycler® 480 (Roche) and Mx3005P® (Agilent), but are also suitable for other real-time PCR instruments. They allow the rapid and sensitive detection of DNA of virulence-associated genes of *E. coli* purified from stool or other sample material (e.g. with the QIAamp DNA Stool Mini Kit). Furthermore, isolated bacterial colonies can be picket from the plate, isolated e.g. with InstaGene™ Matrix (Bio-Rad) and analysed.
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).

Information on kit series:

Assay	Detected gene(s)*	Detection channels	
		Pathogen	IPC
BactoReal® *E. coli* Typing stx1 & stx2 (STEC)	stx1 & stx2	FAM + VIC/HEX	Cy5
BactoReal® *E. coli* Typing eae & ehxA (EHEC, EPEC)	eae & ehxA	FAM + VIC/HEX	Cy5
BactoReal® *E. coli* Typing eae (EHEC or EPEC)	eae	FAM	Cy5
BactoReal® *E. coli* Typing ehxA (EHEC)	ehxA	VIC/HEX	Cy5

*Assays for stx1 (FAM) and stx2 (VIC/HEX), as well as assays for eae (FAM) and ehxA (VIC/HEX) can be combined in a multiplex PCR.

Typing of pathogenic *Escherichia coli* according to virulence-associated genes:

Gene	Detection channel	EPEC	EHEC	STEC
eae	FAM	positive	**eae** *and/or* **ehxA** positive	negative
ehxA	VIC/HEX	negative		negative
stx1	FAM	negative	**stx1** *and/or* **stx2** positive	**stx1** *and/or* **stx2** positive
stx2	VIC/HEX	negative		

eae: intimin **ehxA:** enterohemolysin **stx:** Shiga toxin or Shiga-like toxin (SLT) or verotoxin (vtx)

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. 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 a sequence-specific detection of PCR amplificates.

3. Pathogen information

Escherichia coli is a gram negative bacterium that is a member of the normal gut flora of humans and animals. Most *E. coli* strains are harmless, but some serotypes carry virulence factors which enable them to be pathogenic in target hosts. These pathogenic *E. coli* are implicated in a number of diseases including diarrhoea, septicaemia and urinary tract infections in humans and animals.
Enteropathogenic *Escherichia coli* (EPEC) produces an outer membrane protein called intimin, encoded by *eae* and mediating intestinal cell attachment. Intimin is a virulence factor (adhesin) of EPEC and EHEC strains. Furthermore, EPEC are classified into typical and atypical strains based on the presence of the plasmid *E. coli* adherence factor (EAF). Typical EPEC strains are rarely isolated from animals, and humans are the major natural reservoir. The reservoir for atypical EPEC can be both animals and humans. By definition all EPEC lack genes to produce shiga toxin (stx).
Shiga toxin (verotoxin) producing *Escherichia coli* (STEC) is recognized as an important human diarrheal pathogen. Swine and cattle play an important role as a carrier of STEC. The ability of STEC to cause illness is due to the production of Shiga toxins Stx1 and/or Stx2 (encoded by stx1 and stx2 genes).
Enterohaemorrhagic *Escherichia coli* (EHEC) can cause severe foodborne disease. EHEC is also positive for stx1 and/or stx2 but also produces either intimin (eae) or hemolysin (ehxA).

4. Contents of the Kits

4.1. BactoReal® Kit *E. coli* Typing stx1 & stx2 (STEC)

Labelling	Content	Amount		Storage
		DVEB04013	DVEB04053	
E. coli stx1 Assay Mix (green cap)	Primer and probe (FAM) for detection of stx1	2 x 50 µl	1 x 50 µl	-20°C
E. coli stx2 Assay Mix (purple cap)	Primer and probe (VIC/HEX) for detection of stx2	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
E. coli stx1&2 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)

4.2. BactoReal® Kit *E. coli* Typing eae & ehxA (EHEC, EPEC)

Labelling	Content	Amount		Storage
		DVEB04113	DVEB04153	
E. coli eae Assay Mix (green cap)	Primer and probe (FAM) for detection of eae	2 x 50 µl	1 x 50 µl	-20°C
E. coli ehxA Assay Mix (purple cap)	Primer and probe (VIC/HEX) for detection of ehxA	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
E. coli eae Positive Control (red cap)	Control-DNA (approx. 1,000 target copies/µl)	1 x 25 µl	1 x 25 µl	-20°C
E. coli ehxA 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)

4.3. BactoReal® Kit *E. coli* Typing eae (EHEC or EPEC)

Labelling	Content	Amount		Storage
		DVEB04313	DVEB04353	
E. coli eae Assay Mix (green cap)	Primer and probe (FAM) for detection of eae	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
E. coli eae 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)

4.4 BactoReal® Kit *E. coli* Typing ehxA (EHEC)

Labelling	Content	Amount		Storage
		DVEB04413	DVEB04453	
E. coli ehxA Assay Mix (purple cap)	Primer and probe (VIC/HEX) for detection of ehxA	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
E. coli ehxA 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 *E. coli* Typing are stable until the expiry date stated on the label. Repeated thawing and freezing should be avoided. Please protect kit components from light.

5. 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 *E. coli* 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.

6. Additionally required materials and devices

- Reagents and devices for DNA-extraction
- 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*	2.0-3.0 µl
	DNA Reaction Mix (2x)	10.0 µl
	Assay Mix Assay Mix	1.0 µl each
	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**

*Depending on the amount of assay mix. FAM and VIC/HEX labelled assays can be combined in a multiplex PCR

Performing of multiplex PCR: Assays for stx1 (FAM) and stx2 (VIC/HEX), as well as assays for eae (FAM) and ehxA (VIC/HEX) can be combined in a multiplex PCR.

Positive Control: As positive control please use 1 µl of the according Positive Control(s) each + the appropriate amount of water.

7.2. Programming of the temperature profile

- **Select dyes:** FAM-TAMRA for detection of stx1
  VIC-TAMRA (HEX) for detection of stx2
  FAM-TAMRA for detection of eae
  VIC-TAMRA (HEX) for detection of ehxA
  Cy5-NONE for detection of internal positive control

Select reference dye (passive reference): ROX

Sample Volume: 20 µl

Temperature Profile:

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

For ABI PRISM® 7500:
Ramp speed: Without “fast cycling” parameter

For LightCycler® 480 instrument:
Detection format: 2 or 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.

Table 1: Criteria for a valid interpretation

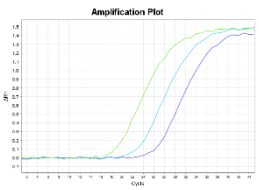
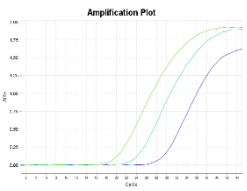
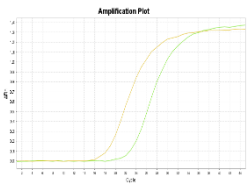
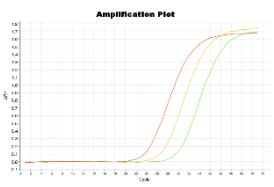
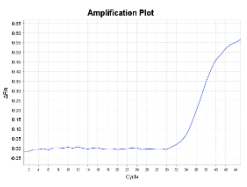
	Ct (FAM) stx1	Ct (VIC/HEX) stx2	Ct (FAM) eae	Ct (VIC/HEX) ehxA	Ct (Cy5) IPC*	Interpretation
Controls						
Extraction negative control	-	-	-	-	26-29	Valid
Negative control	-	-	-	-	26-29	Valid
Positive Control stx1&2	26-29	25-28	-	-	26-29	Valid
Positive Control eae	-	-	26-29	-	26-29	Valid
Positive Control ehxA	-	-	-	26-29	26-29	Valid
Samples						
Sample	-	-	-	-	26-29	Negative for EHEC, EPEC or STEC
Sample	+	+	-	-	26-29/ -	Positive for STEC
Sample	-	+	-	-	26-29/ -	Positive for STEC
Sample	+	-	-	-	26-29/ -	Positive for STEC
Sample	-	-	+	-/+	26-29/ -	Positive for EPEC
Sample	+	+	+	+	26-29/ -	Positive for EHEC
Sample	+	+	+	-	26-29/ -	Positive for EHEC
Sample	+	+	-	+	26-29/ -	Positive for EHEC
Sample	+	-	+	+	26-29/ -	Positive for EHEC
Sample	+	-	+	-	26-29/ -	Positive for EHEC
Sample	+	-	-	+	26-29/ -	Positive for EHEC
Sample	-	+	+	+	26-29/ -	Positive for EHEC
Sample	-	+	+	-	26-29/ -	Positive for EHEC
Sample	-	+	-	+	26-29/ -	Positive for EHEC

*The positive signal of the internal positive control assay excludes a putative PCR inhibition.

For analysis of PCR data please proceed as follows:
For analysis of PCR results gained with BactoReal® Kit *E. coli* Typing please select fluorescence display options FAM channel for the stx1 and eae target, VIC/HEX channel for the stx2 and ehxA target, and Cy5 channel for the internal positive control target. Samples with a positive Cp or Ct-value as shown in Table 1 are considered positive. Please also check the presence of amplification-curves manually.

In case there are no signals in FAM and VIC/HEX channel and no signal with IPC:
→ No interpretation statement can be made.
Information about possible sources of error and their solution can be found in 9. Troubleshooting.

Amplification plots:

Detection of **stx1**	Detection of **stx2**	Detection of **eae**	Detection of **ehxA**	Detection of **IPC**
				
ABI Prism® 7500: FAM channel	**ABI Prism® 7500:** VIC channel	**ABI Prism® 7500:** FAM channel	**ABI Prism® 7500:** VIC channel	**ABI Prism® 7500:** Cy5 channel
1:10 serial dilution of *E. coli* stx1	1:10 serial dilution of *E. coli* stx2	1:10 dilution of *E. coli* eae	1:10 dilution of *E. coli* ehxA	Internal positive control CR-3

9. Troubleshooting

9.1. No specific signal with positive controls:

- 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.

9.2. No signal with the internal positive controls and no *E. coli* 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 volume amount of H_2O).
- Incorrect PCR conditions.
  → Check the PCR conditions and repeat the PCR, if necessary.

9.3. *E. coli* 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.

9.4. *E. coli* 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

Kits were 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 GmbH.

10.1. Analytical sensitivity

The analytical sensitivity is 15 target copies stx1 and stx2 per PCR reaction and approx. 10 target copies eae or ehxA per PCR reaction.

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 EHEC, EPEC or STEC strains. The stx2 variant stx2f is not detected.