BactoReal® Typing Kit *E. coli* v1.1

Manual

For veterinary use only

REF **DVET001**

Σ **2 x 96 wells**

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1. Product description

BactoReal® Typing Kit *E. coli* contains PCR primer sets for the amplification and detection of porcine *E. coli* virulence-associated genes. The method is based on an intercalating dye using real-time PCR. Amplification curve and gene specific melting curve at 515 nm indicate the amplification of *E. coli* specific genes. Screening for porcine *E. coli* adhesion and toxin genes requires picking of several bacterial colonies per plate. With one BactoReal® Typing Kit *E. coli* kit 20 colonies (2 x 96 wells) can be typed.

Mix	primer sets	fimbrial adhesins
1	F4	fimbria F4 (K88)
2	F5	fimbria F5 (K99)
3	F6	fimbria F6 (987P)
4	F18	fimbria F18
5	F41	fimbria F41 (F7)
		enterotoxins
6	STa1	heat-stable enterotoxin (estA)
7	STb2	heat-stable enterotoxin (estB)
8	Stx2e	shiga toxin variant, subtype Stx2e
9	LT	heat-labile enterotoxin (eltB)
10	EAST1	enteroaggregative heat-stable toxin (astA)
		other adhesins
11	fimA	type 1 fimbriae
12	AIDA-I	autotransporter adhesin
13	pAA	porcine attaching and effacing-associated adhesin
14	escV	translocator, type III secretion system, located in pathogenicity island LEE
15	cnf1	cytotoxic necrotizing factor 1
16	iucD	aerobactin
17	papC	pyelonephritis-associated pilus (gene of the P fimbriae for EAEC)
18	pic	serine protease autotransporter

2. Pathogen information

Most types of diarrhea are caused by strains of enterotoxigenic *E. coli* (ETEC). The main virulence factors associated with ETEC in diarrhoea are enterotoxins and fimbrial adhesins. The fimbrial types F4 (K88) and F18 are commonly found in pathogenic *E. coli* isolated from weaned pigs. F18 is most commonly found in Shiga toxin-producing *E. coli* (STEC) and the Stx2e variant has been associated with oedema disease in pigs.
The fimbrial types F5 (K99), F6 (987P) and F41 are more frequently associated with *E. coli* causing neonatal diarrhea. Another virulence factor of ETEC is EAST1, which has also been detected in *E. coli* of different pathogenic strains, such as ETEC, enteropathogenic *E. coli* (EPEC) and STEC from humans and animals.

References:
Wilson RA, Francis DH. Fimbriae and enterotoxins associated with E coli serogroups isolated from clinical cases of porcine colibacillosis. Am J Vet Res. 1986;47:213–217

3. Principle of real-time PCR

A specific DNA sequence of the pathogen genome is amplified by a set of two PCR primers that flank the target region and the generated PCR-product is detected by an intercalating nucleic acid dye with subsequent melt curve analysis.

4. General Precautions

The user should always pay attention to the following:

- Always include a negative control per PCR-run (water instead of sample).
- 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. The 5x HR Master Mix can be stored up to 1 month at room temperature or at 4°C, routine storage is at -20°C.

5. Contents

***E. coli* Assay Mix (green cap)**	**Content Primer for detection of**	**Amount**
Tube 1 and Tube 4	F4 and F18	á 1 x 50 µl
Tube 2 and Tube 11	F5 and fimA	á 1 x 50 µl
Tube 3 and Tube 5	F6 and F41	á 1 x 50 µl
Tube 6 and Tube 12	STa and AIDA	á 1 x 50 µl
Tube 7 and Tube 8	STb and Stx2e	á 1 x 50 µl
Tube 9 and Tube 10	LT and EAST	á 1 x 50 µl
Tube 13	pAA	1 x 50 µl
Tube 14	escV	1 x 50 µl
Tube 15	iucD	1 x 50 µl
Tube 16	cnf1	1 x 50 µl
Tube 17	papC	1 x 50 µl
Tube 18	pic	1 x 50 µl

Labelling (white cap)	**Content**	**Amount**
5x HR Master Mix	Master Mix with internal reference (ROX)	2 x 400 µl

Labelling (red cap)	**Content**	**Amount**
E. coli Positive Control	Control-DNA	1 x 50 µl

The components of BactoReal® Typing *E. coli* should be stored at 4°C to -25°C and are stable until the expiry date stated on the label.

6. Additionally required materials and devices

- Reagents and devices for DNA-extraction
- PCR-grade water
- Disposable powder-free gloves
- Sterile pipette tips with filters
- Real-time PCR instrument
- Appropriate 96 well reaction plates or reaction tubes with corresponding (optical) closing material.

For further information a group-specific typing of *E. coli* can be performed with Ingenetix's BactoReal® Kit *E.coli* Typing which includes a series of different real-time PCR kits for the detection of stx1 and stx2 (BactoReal® Kit *E.coli* Typing STEC), and of eae (intimin) or ehxA (BactoReal® Kit *E.coli* Typing EHEC, EPEC).

7. Preparation of real-time PCR

Please make sure that at least one negative control (water), as well as one positive control (red cap) are included per PCR run.

7.1. Pipetting scheme

Sample: Pick several *E. coli* colonies and isolate the DNA (e.g. with InstaGene™ Matrix, Bio-Rad), the extracted DNA should be used in a dilution (e.g. 1:1000), so that Ct / Cp values of amplification are around 25 cycles.

Positive Control: As positive control use 1 µl of the *E. coli* Positive Control.

Prepare **one** master mix for **each** *E. coli* Assay Mix
or use them in **multiplex**: *E. coli* Assay Mix Tube 1 with 4, Tube 2 with 11,
Tube 3 with 5, Tube 6 with 12,
Tube 7 with 8, Tube 9 with 10, optional Tube 13 with 14:

		Per sample and assay
Preparation of master mix (mix well)	H_2O	14.0 µl
	5x HR Master Mix	4.0 µl
	E. coli Assay Mix	1.0 µl +/- 1.0 µl*
	Total volume	**19.0 µl**
Preparation of PCR	Master mix	19.0 µl
	Sample	1.0 µl
	Total volume	**20.0 µl**

***You don't have to calculate the master mix for the additional µl.

When eight PCRs per colony are on a 96-well plate, prepare the respective master mixes in the rows and transfer with a multichannel pipette on the 96-well plate. Pipette the first sample into the wells A1 to H1 and so on. A total of 10 colonies per plate can be analysed (A11-H11: NTC; A12-H12 Pos. Control.).

If 11-12 different PCRs per colony are run simultaneously on a 96-well plate switch the pipetting scheme by 45° (max. 6 colonies can be analysed).

7.2. Programming of the real-time PCR instrument

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.

Experiment Properties	Reagents: SYBR® Green Reagents
	Ramp speed: Standard
Select dyes	Reporter SYBR, Quencher None
Select passive reference	ROX
Sample Volume	20 µl

Temperature Profile

Holding Stage Cycles: 1 Analysis: None	Holding Stage Cycles: 1 Analysis: None	Cycling Stage Cycles: 35 Analysis: Quantification Acquisition at 60°	Melt Curve Stage Continuous
50°C 2 min	95°C 10 min	95°C 15 sec 60°C 1 min	95°C 15 sec 60°C 1 min 95°C 30 sec 60°C 15 sec

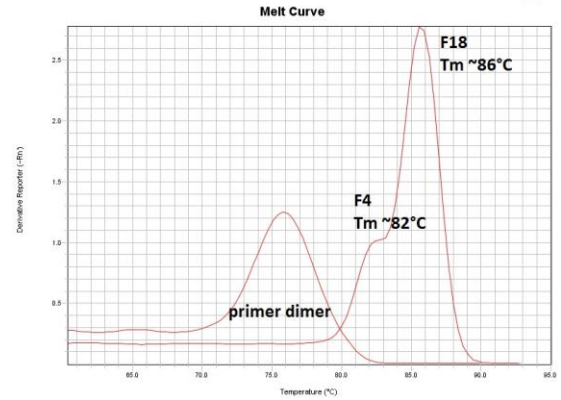
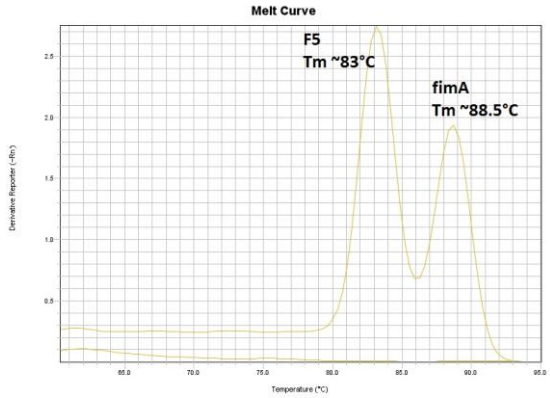
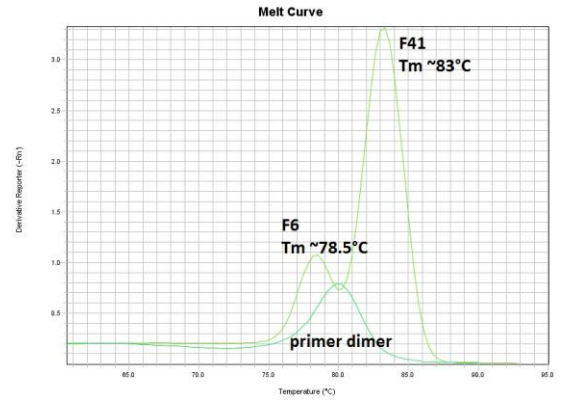
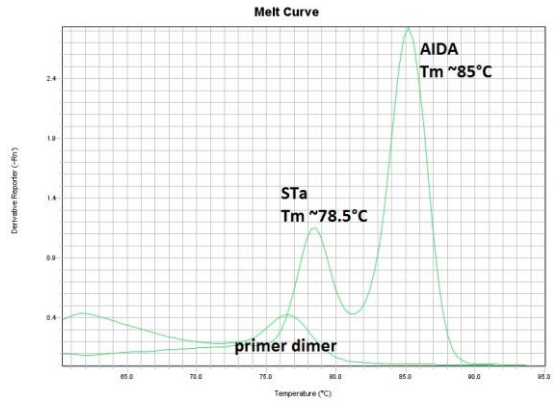
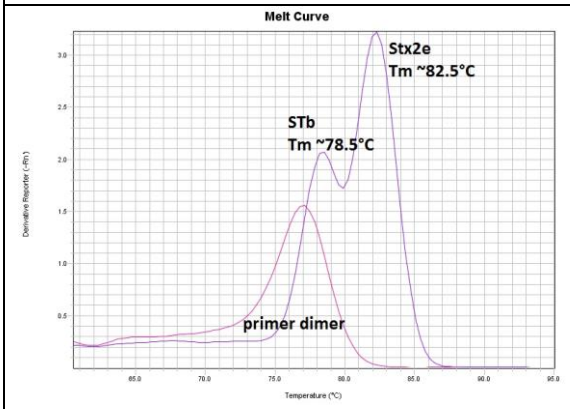
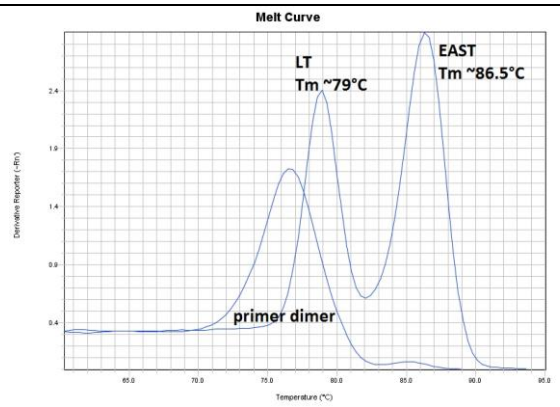
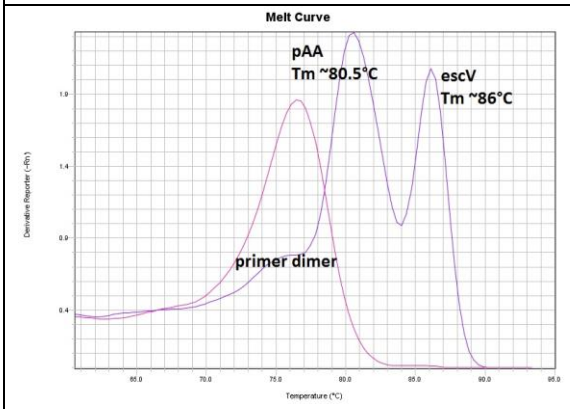
8. Interpretation of Melting Curve data

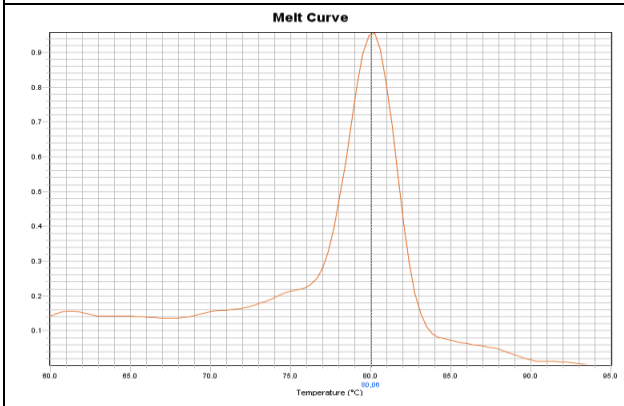
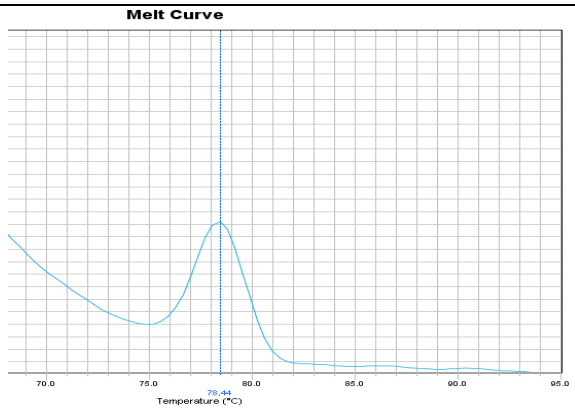
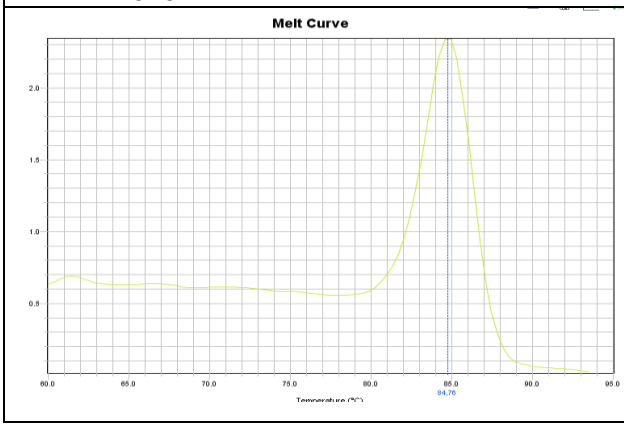
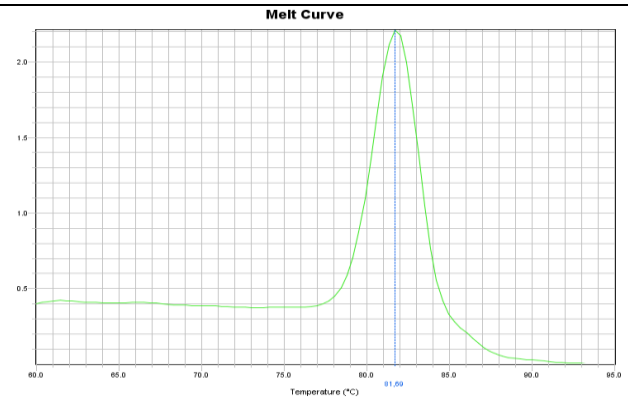
For analysis of PCR results gained with BactoReal® Typing Kit *E. coli* please verify the amplification plot and melt curves.
A specific amplification and melting curve of the sample has to be interpreted in context of the Ct/Cp and Tm [°C] values of the negative control.

	Target 1	Melting Point **Tm** around **[°C]**	Target 2	Melting Point **Tm** around **[°C]**
Tube 1 & 4	**F4**	83.0	**F18**	86.0
Tube 2 &11	**F5**	84.0	**fimA**	89.0
Tube 3 & 5	**F6**	79.5	**F41**	83.0
Tube 6 &12	**STa**	79.5	**AIDA**	85.0
Tube 7 & 8	**STb**	80.0	**Stx2e**	82.5
Tube 9 &10	**LT**	80.5	**EAST**	87.0
Tube 13&14	**pAA**	80.5	**escV**	84.5
Tube 15	**cnf1**	80.0		
Tube 16	**iucD**	80.0		
Tube 17	**papC**	85.0		
Tube 18	**pic**	82.0		

Annotation: Due to sequence variation and other factors a shift of melting point Tm can be observed.

It is possible that due to a shift of Tm the two melting points can't be identified in the multiplex PCRs. Please set up the assay mix as singleplex reaction.

Tube 1&4: F4, F18	Tube 2&11: F5, fimA
	
Tube 3&5: F6, F41	**Tube 6&12: STa, AIDA**
	
Tube 7&8: STb, Stx2e	**Tube 9&10: LT, EAST**
	
Tube 13&14: pAA, escV	
	

Tube 15: cnf1	Tube 16: iucD
	
Tube 17: papC	**Tube 18: pic**
	

Melt curve analysis was performed on an Applied Biosystems® 7500 instrument (Thermo Fisher Scientific).

Annotation: Due to the multiplexing of primers a formation of primer dimers can be observed.

9. Annex – Symbols

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

Permitted Use: The 5x HR Master Mix contains 5x HOT FIREPol® SolisGreen qPCR Mix which is a product of Solis BioDyne OÜ of 51014 Tartu, Estonia (www.sbd.ee). CYGREEN dye is a component of this qPCR Master Mix. CYGREEN is a U.S. registered trademark of Enzo Life Sciences, Inc. U.S. Patent Nos. 8,153,802 and 7,569,695 CYGREEN is a U.S. Registered trademark of Enzo Life Sciences, Inc. and this dye is the subject of the U.S. patents 12,456,502 and 7,569,695.