BactoReal® Kit Footrot

Manual

For veterinary use only

REF **DVEB06813** Σ **100**

REF **DVEB06853** 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. Intended use

BactoReal® Kit Footrot is a kit, based on real-time PCR, for the detection of *Fusobacter necrophorum* subsp. *necrophorum* and *Dichelobacter nodosus.*

2. Product description

BactoReal® Kit Footrot contains primers and probes for the amplification and detection of the polynucleotide phosphorylase (pnpA) gene of *Dichelobacter nodosus* and the gyrase subunit B (gyrB) gene of *Fusobacter necrophorum* subsp. *necrophorum*, further a positive control, the amplification mix and an internal positive control. This test has been developed for use with the Applied Biosystems® (ABI) 7500 instrument (Thermo Fisher Scientific), LightCycler® 480 II (Roche) and Mx3005P® (Agilent), but is designed for compatibility with most real-time PCR instruments except capillary instruments. The kit allows rapid and sensitive detection of DNA of *Fusobacter necrophorum* subsp. *necrophorum* and *Dichelobacter nodosus* in samples purified from biopsies and swabs (e.g. with the QIAamp® DNA Mini Kit, Qiagen).

BactoReal® Kit Footrot detects the pnpA gene of *Dichelobacter nodosus* and the gyrB gene of *Fusobacter necrophorum* subsp. *necrophorum.* A probe-specific amplification curve at 530 nm (FAM channel) indicates the amplification of *D. nodosus*-specific DNA. A probe-specific amplification curve at 554 nm (VIC/HEX channel) indicates the amplification of *F. necrophorum*-specific DNA.
The kit contains an internal positive control system (IPC) for detection in Cy5 channel (667 nm) excluding false-negative results due to inhibition of real-time PCR.

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

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

3. Pathogen information

Footrot (infectious pododermatitis), is a hoof infection commonly found in sheep, goats, and cattle. It is responsible for the majority of cases of lameness in sheep. In sheep, there are two types of bacteria involved in causing footrot. *Dichelobacter nodosus* is the main cause of the disease, while *Fusobacterium necrophorum* is an associated pathogen. The two usually have a synergistic effect together. *D. nodosus* is an anaerobic, non-sporulating, gram-negative bacterium. *F. necrophorum*, which is always present in the atmosphere, is an obligate anaerob, gram-negative bacterium and produces a number of exotoxins that damage the hoof of the animal. *Fusobacterium necrophorum* is classified into subsp. *necrophorum* and subsp. *funduliforme*. Replication of the bacteria and disease transmission usually occur under humid and hot weather conditions that are typical of the rainy season.

References:

Luci A. Witcomb, Laura E. Green, Leo A. Calvo-Bado, Claire L. Russell, Edward M. Smith, Rose Grogono-Thomas, and Elizabeth M.H. Wellington. 2015. First study of pathogen load and localisation of ovine footrot using fluorescence in situ hybridisation (FISH). Vet. Microbiol. 176(3-4): 321–327.

4. General Precautions

The user should 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 is included during DNA-extraction (for example extraction of water instead of sample material), in order to exclude false-positive results due to contamination with *D. nodosus* and *F. necrophorum* 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 aerosol barrier pipette tips.
- Thaw all components thoroughly at room temperature before pipetting. When thawed, mix the components and centrifuge briefly.
- For MSDS, see www.ingenetix.com.

5. Additionally required materials and devices

- Aerosol barrier pipette tips
- Appropriate optical 96-well reaction plates or reaction tubes with optical closing material recommended by the manufacturer of the real-time PCR instrument
- Real-time PCR instrument which is able to detect and differentiate fluorescence in FAM, VIC and Cy5 channel

Examples of real-time PCR instruments with required dye channels:

Instruments with **FAM** (510 nm), **VIC** (554 nm) and **Cy5** (667 nm) channels:

e.g. ABI® 7500, QuantStudio™ 5 or 6 with the correct color calibration (Thermo Fisher Scientific), Mx3005P® (Agilent), LightCycler® 480 II or Cobas z 480 (Roche), Rotor-Gene Q 5plex (QIAGEN), CFX96 (BioRad) or qTOWER module 1&2&5 (Analytik Jena)

6. Contents of the Kit

6.1. Order Numbers BactoReal® Kit Footrot

Order Number	Reactions	Dye Channel Pathogen	Dye Channel IPC
DVEB06811	100	FAM + VIC	Cy5 (CR-3 Assay)
DVEB06851	50		

6.2 Content

Component	Content	Quantity		Storage
		100 rxn	50 rxn	
Footrot Assay Mix (green cap)	Primer and probe for detection of *D. nodosus* (FAM) and *F. necrophorum* (VIC)	2 x 50 µl	1 x 50 µl	-15°C to -25°C
CR3 Assay Mix (yellow cap)	Primer, probe (Cy5) and target for detection of IPC	2 x 50 µl	1 x 50 µl	-15°C to -25°C
Footrot Positive Control (red cap)	Control-DNA (approx. 10,000 target copies/µl)	1 x 25 µl	1 x 25 µl	-15°C to -25°C
DNA Reaction Mix (white cap)	Reaction Mix	2 x 500 µl	1 x 500 µl	-15°C to -25°C until first use, then at +4°C
Water (blue cap)	Water	1 x 1000 µl	1 x 1000 µl	-25°C to +4°C

The components of BactoReal® Kit Footrot are stable until the expiry date stated on the label. Repeated freeze/thaw cycles should be avoided. Protect kit components from light.

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.

7. Preparation of real-time PCR

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 pathogen detection 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
	Footrot Assay Mix	1.0 µl
	CR3 Assay Mix	1.0 µl
	Total volume Master Mix	15.0 µl
Preparation of PCR assay	Master mix	15.0 µl
	Sample*	5.0 µl
	Total volume	20.0 µl

*1-8 µl sample can be used. When using a volume other than 5 µl, the volume of H_2O has to be adjusted accordingly.

Positive Control: Use 1 µl of Footrot Positive Control + 4 µl H_2O.

7.2. Programming of the temperature profile

Further information on programming the real-time PCR instrument can be found in the respective operator's manual. Keep in mind that some PCR-platforms first have to be calibrated with the corresponding dye before performing multiplex-PCR.

Select dyes: FAM-TAMRA for detection of *D. nodosus*
VIC-NONE for detection of *F. necrophorum*
Cy5-NONE (CR-3 Assay Mix) for detection of IPC

Select passive reference dye: ROX

Sample Volume: 20 µl

Temperature Profile:

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

<u>For Applied Biosystems® 7500</u>
Ramp speed: Without "fast cycling" parameter

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

*) UNG (Uracil-N-glycosylase) is a component of the DNA Reaction Mix as are dNTPs with dUTP to eliminate future amplicon carryover. Since the native template does not contain dUTP, it remains intact in the presence of the enzyme. The UNG enzyme eliminates contamination by any amplicon DNA that contains dUTP from a previous reaction by excising uracil residues from DNA cleaving the N-glycosylic bond creating abasic sites that do not serve as good DNA templates for Taq polymerase.

****Note:** If viral RNA is 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®, ViroReal®, MycoReal and ParoReal kits on all PCR instruments.

8. Interpretation of PCR-data

For analysis of PCR results gained with BactoReal® Kit Footrot, select fluorescence display options FAM channel for the *D. nodosus* target, VIC channel for the *F. necrophorum* target and Cy5 channel for the IPC. Samples with positive Cp or Ct-values are considered positive. Please, check amplification curves and adjust the threshold manually, if necessary. Samples should be inspected both in logarithmic and linear scale view and compared with the negative control.

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

	Ct/Cp (FAM) ***D. nodosus*** **target**	**Ct/Cp (VIC)** ***F. necrophorum*** **target**	**Ct/Cp[1)] (Cy5) IPC target**	**Interpretation**
Negative control	Negative	Negative	Positive	Valid
Positive control (undiluted, 1 µl/PCR)	Positive[1)]	Positive[1)]	Positive	Valid
Extraction negative control (optional)	Negative	Negative	Positive	Valid
Negative sample	Negative	Negative	Positive	Valid
Positive sample	Positive	Positive	Positive/Negative[2)]	Valid

1) The calculated Ct-value can vary depending on the real-time PCR instrument and software used. Ct-values of positive control and IPC are expected at ~ Ct 30.

   Always verify and compare IPC Ct-values with respect to shape and increment of the fluorescence reporter signal of the amplification curve to exclude inhibition of the reaction.

2) High pathogen load in the sample can lead to a reduced or absent fluorescence signal of the IPC.

8.1. Signal in FAM channel

→ DNA of *Dichelobacter nodosus* has been amplified. The sample has to be interpreted as positive for *D. nodosus*.

8.2. Signal in VIC channel

→ DNA of *Fusobacter necrophorum* subsp. *necrophorum* has been amplified. The sample has to be interpreted as positive for *F. necrophorum*.

8.2. No signal in FAM or VIC channel but signal with IPC

→ No *D. nodosus* or *F. necrophorum* DNA is detectable in the sample. The sample has to be interpreted as negative.
The positive signal of the IPC excludes a putative PCR inhibition.

8.3. No signals in FAM or VIC channel and no signal with IPC

→ No interpretation can be made.
Information about possible error sources and their solution can be found in 9. Troubleshooting.

9. Troubleshooting

9.1. No *D. nodosus* and *F. necrophorum* 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.

9.2. No signal with IPC and no *D. nodosus* and *F. necrophorum* specific signal with sample

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 volume of H_2O).

Incorrect PCR conditions.
→ Check the PCR conditions and repeat the PCR, if necessary.

9.3. *D. nodosus* or *F. necrophorum* specific signal with 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 workspace and instruments are decontaminated at regular intervals.

9.4. *D. nodosus* or *F. necrophorum* specific signal with negative control of extraction (optional)

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

10. Specifications and performance evaluation

BactoReal® Kit Footrot has been evaluated with an ABI® 7500 instrument (Thermo Fisher Scientific). For further validation data contact ingenetix.

10.1. Analytical sensitivity and linearity

BactoReal® Kit Footrot has been tested with a 10-fold dilution series of DNA containing fragments of *D. nodosus* and *F. necrophorum* DNA. Analytical sensitivity is approx. 50 target copies/PCR reaction.

For *D. nodosus*, the assay shows **linearity** over the range of 10,000 to 10,000,000 target copies/reaction with a slope of -4.2 and a R^2 of 1.0 as shown in Figure 1.

For *F. necrophorum*, the assay shows **linearity** over the range of 10,000 to 10,000,000 target copies/reaction with a slope of -3.7 and a R^2 of 0.997 as shown in Figure 2.

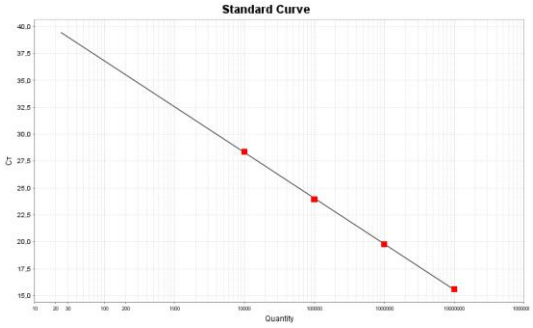

Figure 1 Ten-fold dilution series of a *D. nodosus* DNA standard plotted against Ct

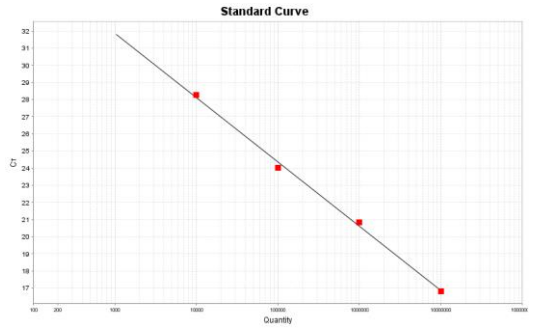

Figure 2 Ten-fold dilution series of a *F. necrophorum* DNA standard plotted against Ct

10.2. Analytical specificity

The specificity is ensured by the selection of highly specific primers and probes. Primers and probes have been checked for possible homologies to currently published sequences by sequence comparison analyses. This also validated the detection of so far known *D. nodosus* and *F. necrophorum* strains. The kit is specific for *Dichelobacter nodosus* and *Fusobacterium necrophorum* subsp. *necrophorum*. It does not detect the subspecies *Fusobacterium necrophorum* subsp. *funduliforme*, which is generally considered to be less virulent.

10.3. Kit performance

Performance of BactoReal® Kit Footrot with an ABI® 7500 instrument is shown in Figure 3.

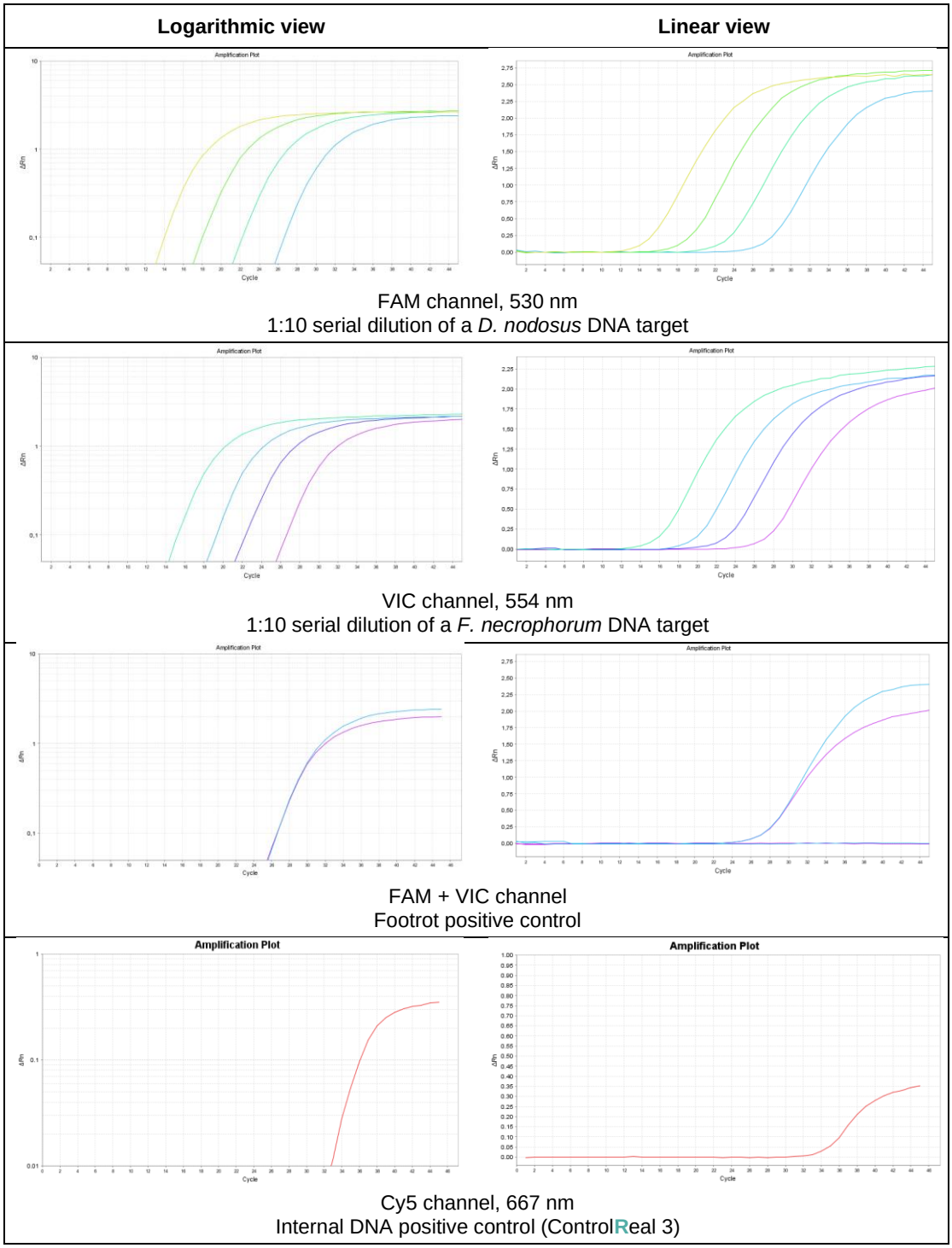

Figure 3 Performance of BactoReal® Kit Footrot