BactoReal® Kit European Foulbrood

Manual

For use with the

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

For veterinary use only

REF **DVEB05513, DVEB05511** Σ **100**

REF **DVEB05553, DVEB05551** Σ **50**

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1. Product description

BactoReal® Kit European Foulbrood is a real-time PCR assay for detection of *Melissococcus plutonius* DNA. This test was developed and validated for the ABI PRISM® 7500 (Fast) instrument (Life Technologies), LightCycler® 480 (Roche), and for Mx3005P® (Agilent) but is also suitable for other real-time PCR instruments. This test allows the rapid and sensitive detection of DNA of *Melissococcus plutonius* from samples purified from swabs, tissues, etc. (e.g. with the QIAamp DNA Mini Kit).

BactoReal® Kit European Foulbrood detects the 16S rRNA gene of *Melissococcus plutonius*. A probe-specific amplification-curve at 530 nm (FAM channel) indicates the amplification of *M. plutonius* specific DNA.
An internal positive control system for detection in VIC/HEX channel (554 nm, order no. DVEB05511 or DVEB05551) or Cy5 channel (667 nm; order no. DVEB05513 or DVEB05553) 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

European foulbrood (EFB) is a bacterial brood disease of honey bees caused by the Gram-positive bacterium *Melissococcus plutonius.* The disease occurs throughout the world. It may cause serious losses of brood. Larvae get infected by consuming contaminated food fed by the nurse bees. The bacteria multiply within the larval gut, competing with it for its food. Larvae that die from the disease have been starved of food.

References:
Eva Forsgren, Giles E Budge, Jean-Daniel Charrière and Michael A Z Hornitzky. 2013. Standard methods for European foulbrood Research. Journal of Apicultural Research 52(1):1-14.

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 *M. plutonius* 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

5.1. BactoReal® Kit order European Foulbrood no. DVEB05511 or DVEB05551

Labelling	Content	Amount		Storage
		DVEB05511	DVEB05551	
EFB Assay Mix (green cap)	Primer and probe (FAM) for detection of *M. plutonius*	2 x 50 µl	1 x 50 µl	-20°C
CR-1 Assay Mix (yellow cap)	Primer, probe (VIC/HEX) and target for detection of IPC	2 x 50 µl	1 x 50 µl	-20°C
EFB 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)

5.2. BactoReal® Kit order European Foulbrood no. DVEB05513 or DVEB05553

Labelling	Content	Amount		Storage
		DVEB05513	DVEB05553	
EFB Assay Mix (green cap)	Primer and probe (FAM) for detection of *M. plutonius*	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
EFB 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

The components of BactoReal® Kit European Foulbrood 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 and VIC/HEX or 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
	EFB Assay Mix	1.0 µl
	CR 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 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 please use 1 µl of the EFB 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: FAM-TAMRA for detection of *M. plutonius*
Cy5-NONE (CR-3 Assay Mix) **or** VIC-TAMRA (CR-1 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 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

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

<u>For LightCycler® 480 instrument:</u>
Detection format: 2 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 (FAM channel) *M. plutonius* target	Ct/Cp IPC target	Interpretation
Negative control	Negative	36.0 ± 2	Valid
Positive control (undiluted, 1 µl/PCR)	28.0-31.0	36.0 ± 2	Valid
Or: positive control (1:10 diluted, 1 µl/PCR)	31.0-34.0	36.0 ± 2	Valid
Extraction negative control (optional)	Negative	36.0 ± 2	Valid
Negative sample	Negative	36.0 ± 2	Valid
Positive sample	Positive	Positive/Negative	Valid

For analysis of PCR data please proceed as follows:
For analysis of PCR results gained with BactoReal® Kit European Foulbrood please select fluorescence display options FAM channel for the *M. plutonius* target and VIC/HEX channel (order no. DVEB05511, DVEB05551) or Cy5 channel (order no. DVEB05513, DVEB05553) for the internal positive control target. Samples with a positive Cp or Ct-value are considered positive. Please also check the presence of amplification-curves manually.

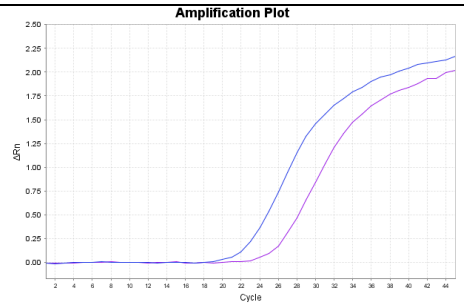
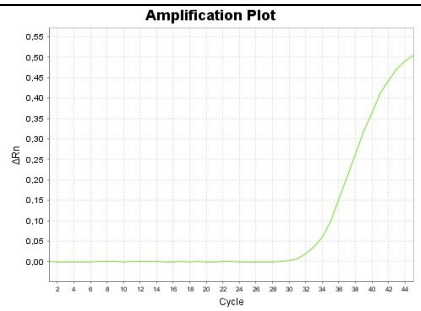
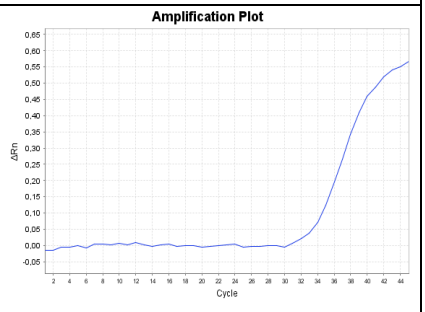
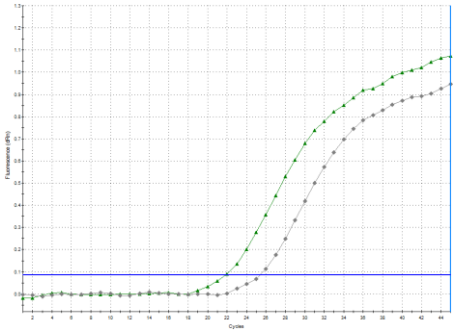
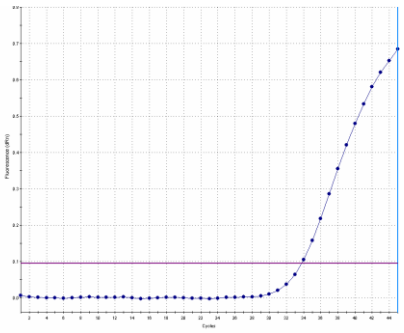
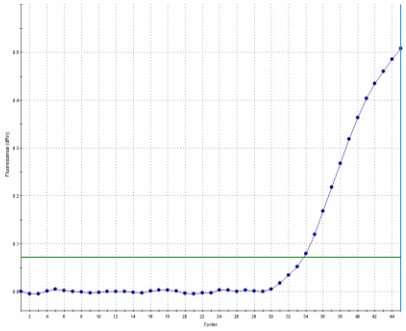
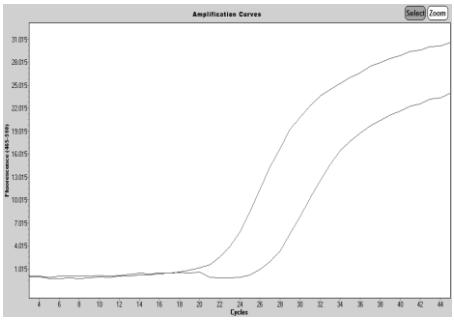
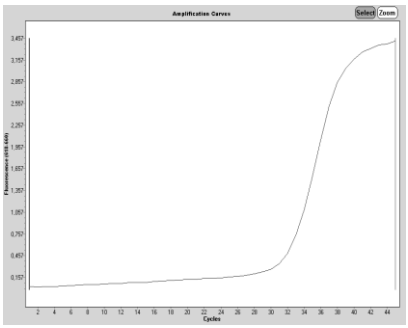
Once the analysis is completed, the following results are possible:

1. Signal in FAM channel:
→ DNA of *M. plutonius* was amplified. The sample has to be interpreted as positive.
M. plutonius DNA can lead to a reduced or absent fluorescence signal of the internal positive control (competition of PCR).

2. No signal in FAM channel:
→ No *M. plutonius* DNA is detectable in the sample. The sample has to be interpreted as negative.
An inhibition of PCR cannot be excluded.

2a. No signal in FAM channel but signal of the internal positive control:
→ No *M. plutonius* 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.

2b. No signals in FAM channel and no signal with internal positive control:
→ No interpretation statement can be made.
Information about possible sources of error and their solution can be found in 9. Troubleshooting.

Detection of ***Melissococcus plutonius***	Detection of internal positive control **CR-3**	Detection of internal positive control **CR-1**
		
ABI Prism® 7500: FAM channel, 530 nm 1:10 dilution of *Melissococcus plutonius* DNA	**ABI Prism® 7500:** Cy5 channel, 667 nm Internal positive control	**ABI Prism® 7500:** VIC channel, 554 nm Internal positive control
		
Mx3005P®: FAM channel 1:10 dilution of *Melissococcus plutonius* DNA	**Mx3005P®:** CY5 channel Internal positive control	**Mx3005P®:** HEX channel Internal positive control
		
LightCycler® 480: FAM channel 1:10 dilution of *Melissococcus plutonius* DNA	**LightCycler® 480:** Cy5 channel Internal positive control	

9. Troubleshooting

1. No *M. plutonius* 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 *M. plutonius* 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. *M. plutonius* 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. *M. plutonius* 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 European foulbrood 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 is 5 target copies/PCR reaction. The limit of detection (LoD95 = smallest number of copies of target DNA which can be detected in 95% of cases) of 36 target copies/reaction 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 *M. plutonius* strains.

11. Annex – symbols

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