BactoReal® Kit American Foulbrood 1.1

Manual

For use with the

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

For veterinary use only

REF **DVEB05411, DVEB05413** Σ **100**

REF **DVEB05451, DVEB05453** Σ **50**

ingenetix GmbH
Arsenalstr.11
1030 Vienna, Austria
T +43 (0)1 36 198 0 198
F +43 (0)1 36 198 0 199
office@ingenetix.com
www.ingenetix.com

Index

1. Product description

BactoReal® Kit American Foulbrood 1.1 is a real-time PCR assay for detection of *Paenibacillus larvae* DNA. This test was developed and validated for the ABI PRISM® 7500 (Fast) instrument ((Thermo Fisher Scientific), 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 *Paenibacillus larvae* from samples purified from swabs, tissues, etc. (e.g. with the QIAamp DNA Mini Kit).

BactoReal® Kit American Foulbrood 1.1 detects the 16S rRNA gene of *Paenibacillus larvae*. A probe-specific amplification-curve at 530 nm (FAM channel) indicates the amplification of *Paenibacillus larvae* specific DNA.
An internal positive control system for detection in VIC/HEX channel, (554 nm, order no. DVEB05411 or DVEB05451) or Cy5 channel (667 nm; order no. DVEB05413 or DVEB05453) 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

American foulbrood (AFB) is a bacterial brood disease of honey bees caused by the Gram-positive and spore-forming bacterium *Paenibacillus larvae.* The disease is transferred and initiated only by the spore stage of the bacterium. Young bee larvae are infected by spores, then the spores germinate in the gut of the larva and the vegetative form of the bacteria begins to grow, taking its nourishment from the larva. Infected larvae normally die after their cell is sealed. The vegetative form of the bacterium produces many millions of spores which may remain dormant for many years.

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 *P. larvae* 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 American Foulbrood 1.1 order no. DVEB05411 or DVEB05451

Labelling	Content	Amount		Storage
		DVEB05411	DVEB05451	
AFB Assay Mix 1.1 (green cap)	Primer and probe (FAM) for detection of *P. larvae* spp.	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
AFB 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 American Foulbrood 1.1 order no. DVEB05413 or DVEB05453

Labelling	Content	Amount		Storage
		DVEB05413	DVEB05453	
P. larvae Assay Mix 1.1 (green cap)	Primer and probe (FAM) for detection of *P. larvae* spp.	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
AFB 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 American Foulbrood 1.1 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
	AFB Assay Mix 1.1	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.

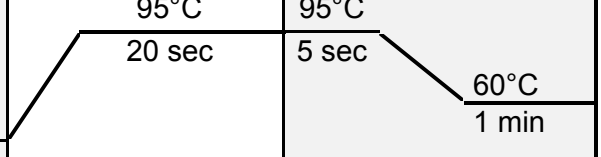
Positive Control: As positive control please use 1 µl of the AFB Positive Control + 4 µl H_2O.
Optional: a 1:10 dilution of the positive control can be used and defined as second standard value (approx. 1000 target copies/µl).

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 *P. larvae*
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



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

For LightCycler® 480 instrument:
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) *P. larvae* target	Ct/Cp IPC target	Interpretation
Negative control	Negative	32.0 ± 2	Valid
Positive control (undiluted, 1 µl/PCR)	28.0-31.0	32.0 ± 2	Valid
Or: positive control (1:10 diluted, 1 µl/PCR)	31.0-34.0	32.0 ± 2	Valid
Extraction negative control (optional)	Negative	32.0 ± 2	Valid
Negative sample	Negative	32.0 ± 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 American Foulbrood 1.1 please select fluorescence display options FAM channel for the *P. larvae* target and VIC/HEX channel (order no. DVEB05411, DVEB05451) or Cy5 channel (order no. DVEB05413, DVEB05453) 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.

8.1. Signal in FAM channel

→ DNA of *P. larvae* was amplified. The sample has to be interpreted as positive.
P. larvae DNA can lead to a reduced or absent fluorescence signal of the internal positive control (competition of PCR).

8.2. No signal in FAM channel but signal of the internal positive control

→ No *P. larvae* 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. However, IPC Ct-values should show comparable results. A shift of Ct-values can indicate a partial inhibition of PCR.

8.3. 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.

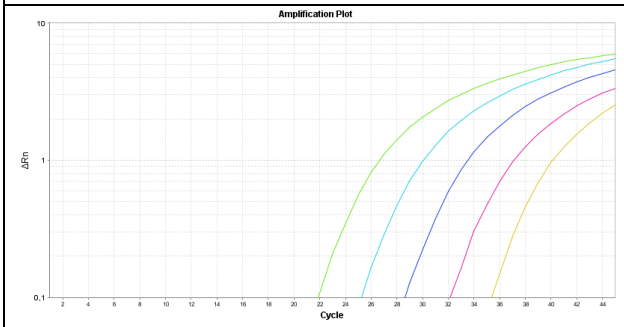
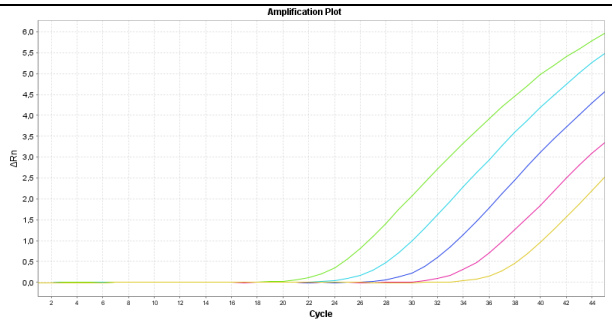
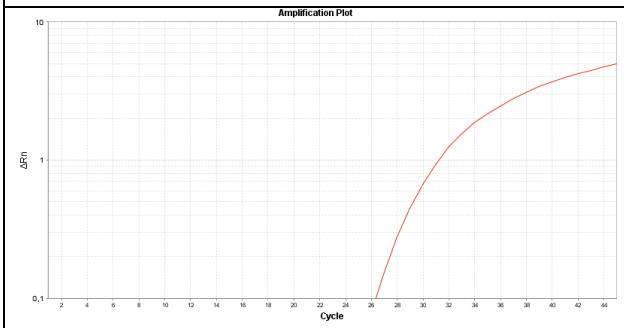
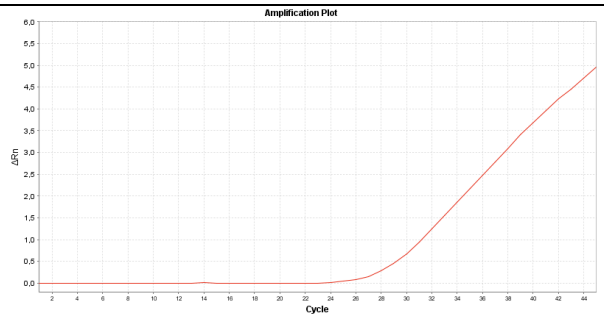
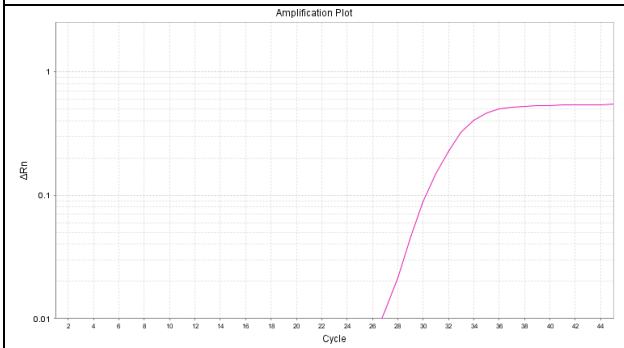
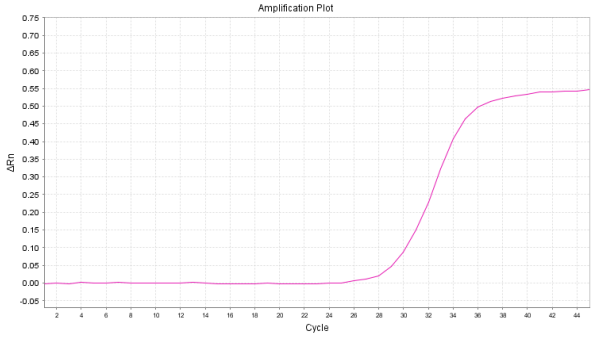
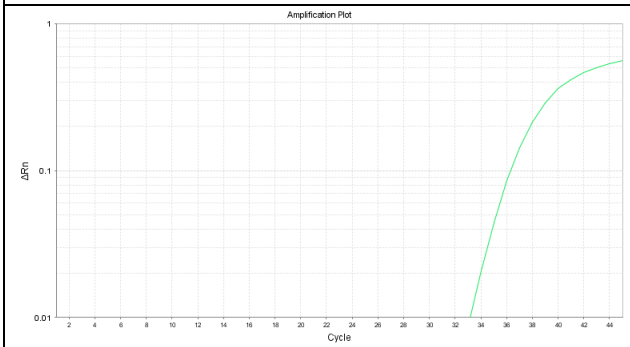
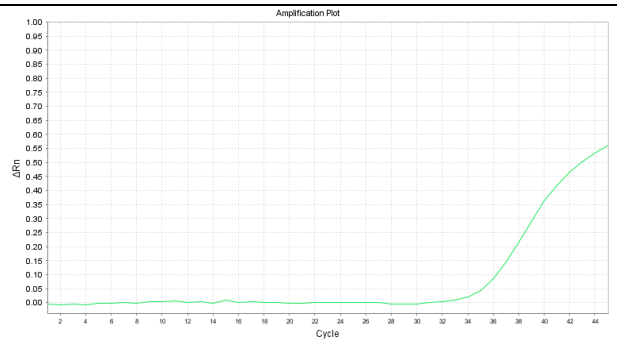
Logarithmic view	Linear view
	
ABI Prism® 7500: FAM channel, 530 nm 1:10 serial dilution of a *P. larvae* DNA target	
	
ABI Prism® 7500: FAM channel, 530 nm *P. larvae* (AFB) positive control	
	
ABI Prism® 7500: Cy5 channel, 667 nm Internal DNA positive control	
	
ABI Prism® 7500: VIC/HEX channel, 554 nm Internal DNA positive control	

Figure 1 Performance of BactoReal® Kit American Foulbrood 1.1

9. Troubleshooting

1. No *P. larvae* 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 IPC and no *P. larvae* specific signal with 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. *P. larvae* 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 work space and instruments are decontaminated at regular intervals.

4. *P. larvae* specific signal with 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 American Foulbrood 1.1 was evaluated with the ABI PRISM® 7500 (Fast) instrument (Thermo Fisher Scientific), with the LightCycler® 480 (Roche) and the Mx3005P® (Agilent). For further validation data please contact ingenetix.

10.1. Analytical sensitivity

The analytical sensitivity is 40 target copies/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 *P. larvae* strains.

11. Annex – symbols

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