

MycoReal[®] Kit

Batrachochytrium dendrobatidis

Manual

For use with the

- ABI PRISM[®] 7500 (Fast)
- Mx3005P[®]
- LightCycler[®] 480



For veterinary use only

REF DVEF00311, DVEF00313		100
REF DVEF00351, DVEF00353		50



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Explanation of symbols



Batch code



Catalogue number



Contains sufficient for <n> tests



Use by



Manufactured by



Store at

1. Product description

Mycoreal Kit *Batrachochytrium dendrobatidis* is a real-time PCR kit for detection of *Batrachochytrium dendrobatidis* DNA. This test was developed 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 *Batrachochytrium dendrobatidis* from samples purified from tissues and swabs (e.g. with the QIAamp DNA Mini Kit).

Mycoreal Kit *Batrachochytrium dendrobatidis* detects the ITS2 region of *Batrachochytrium dendrobatidis*. A probe-specific amplification-curve at 530 nm (FAM channel) indicates the amplification of *B. dendrobatidis* specific DNA.

An internal positive control system (IPC) for detection in VIC/HEX channel, (554 nm, order no. DVEF00311 or DVEF00351) or Cy5 channel (667 nm; order no. DVEF00313 or DVEF00353) 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 of a multiplex-PCR.

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

2. Pathogen information

Batrachochytrium dendrobatidis, also known as the amphibian chytrid fungus, causes the disease chytridiomycosis in amphibians. It infects the keratinized skin of amphibians.

References:

Piotrowski JS, Annis SL, Longcore JF (2004). Physiology of *Batrachochytrium dendrobatidis*, a chytrid pathogen of amphibians. *Mycologia* 96:9-15

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 amplicates.

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 *B. dendrobatidis* 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. MycoReal Kit *Batrachochytrium dendrobatidis* order no. DVEF00311 or DVEF00351

Labelling	Content	Amount		Storage
		DVEF00311	DVEF00351	
<i>Batrachochytrium dendrobatidis</i> Assay Mix (green cap)	Primer and probe (FAM) for detection of <i>B. dendrobatidis</i>	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
<i>Batrachochytrium dendrobatidis</i> 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. MycoReal Kit *Batrachochytrium dendrobatidis* order no. DVEF00313 or DVEF00353

Labelling	Content	Amount		Storage
		DVEF00313	DVEF00353	
<i>Batrachochytrium dendrobatidis</i> Assay Mix (green cap)	Primer and probe (FAM) for detection of <i>B. dendrobatidis</i>	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
<i>Batrachochytrium dendrobatidis</i> 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 MycoReal Kit *Batrachochytrium dendrobatidis* 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
	<i>Batrachochytrium dendrobatidis</i> 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 a volume other than 5 µl, the volume of H₂O has to be changed accordingly.

Positive Control: As positive control use 1 µl of the *Batrachochytrium dendrobatidis* Positive Control + 4 µl H₂O.

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 of a multiplex-PCR.

Select dyes: FAM-TAMRA for detection of *B. dendrobatidis*

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 ingenetix ViroReal®, BactoReal®, MycoReal and ParoReal 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) <i>B. dendrobatidis</i> target	Ct/Cp IPC target	Interpretation
Negative control	Negative	36.0 ± 2	Valid
Positive control (undiluted, 1 µl/PCR)	29.0-32.0	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 MycoReal Kit *Batrachochytrium dendrobatidis* please select fluorescence display options FAM channel for the *B. dendrobatidis* target and VIC/HEX channel (order no. DVEF00311, DVEF00351) or Cy5 channel (order no. DVEF00313, DVEF00353) for the internal positive control target (IPC). Samples with a positive Cp or Ct-value are considered positive. Please also check amplification-curves manually.

8.1. Signal in FAM channel:

→ DNA of *B. dendrobatidis* was amplified. The sample has to be interpreted as positive.
Batrachochytrium dendrobatidis DNA can lead to a reduced or absent fluorescence signal of the IPC.

8.2. No signal in FAM channel but signal of the IPC:

→ No *B. dendrobatidis* DNA is detectable in the sample. The sample has to be interpreted as negative.
The positive signal of the IPC assay excludes a putative PCR inhibition.

8.3. No signals in FAM channel and no signal with the IPC:

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

9. Troubleshooting

9.1. No *B. dendrobatidis* 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 the IPC and no *B. dendrobatidis* 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 volume of H₂O).
- Incorrect PCR conditions.
→ Check the PCR conditions and repeat the PCR, if necessary.

9.3. *B. dendrobatidis* 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. *B. dendrobatidis* specific signal with the negative control of extraction (optional):

- 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 and performance evaluation

Mycoreal Kit *Batrachochytrium dendrobatidis* was evaluated with the ABI PRISM® 7500 (Fast) instrument (Thermo Fisher Scientific). For further validation data please contact ingenetix GmbH.

10.1. Analytical sensitivity and linearity

Mycoreal Kit *Batrachochytrium dendrobatidis* was tested with a 10-fold dilution series of a plasmid containing a fragment of *Batrachochytrium dendrobatidis* DNA. At least 10 target copies/PCR reaction could be detected.

The assay shows **linearity** over the range of 100 to 10,000,000 target copies/reaction with a slope of -3.4 and a R_2 of > 0.99 as shown in Figure 1.

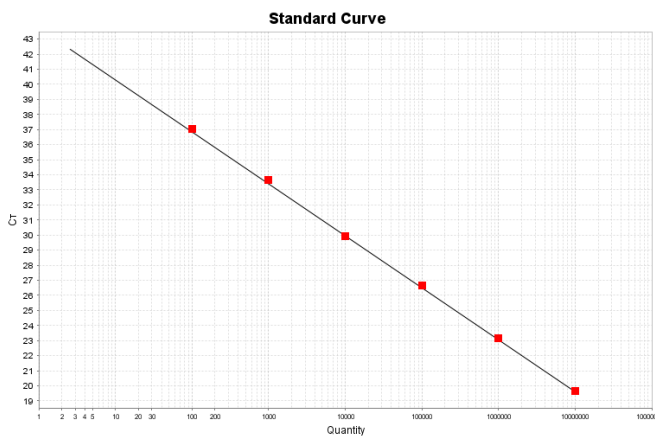


Figure 1 Ten-fold dilution series of a *B. dendrobatidis* DNA standard plotted against CT

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 *Batrachochytrium dendrobatidis* strains.

Furthermore, 89 field samples were tested. Out of these, 82 samples were positive for *B. dendrobatidis* and 7 negative.

10.3. Kit performance

Performance of MycoReal Kit *Batrachochytrium dendrobatidis* with an Applied Biosystems® 7500 Fast Real-time PCR System (Thermo Fisher Scientific) is shown in Figure 2.

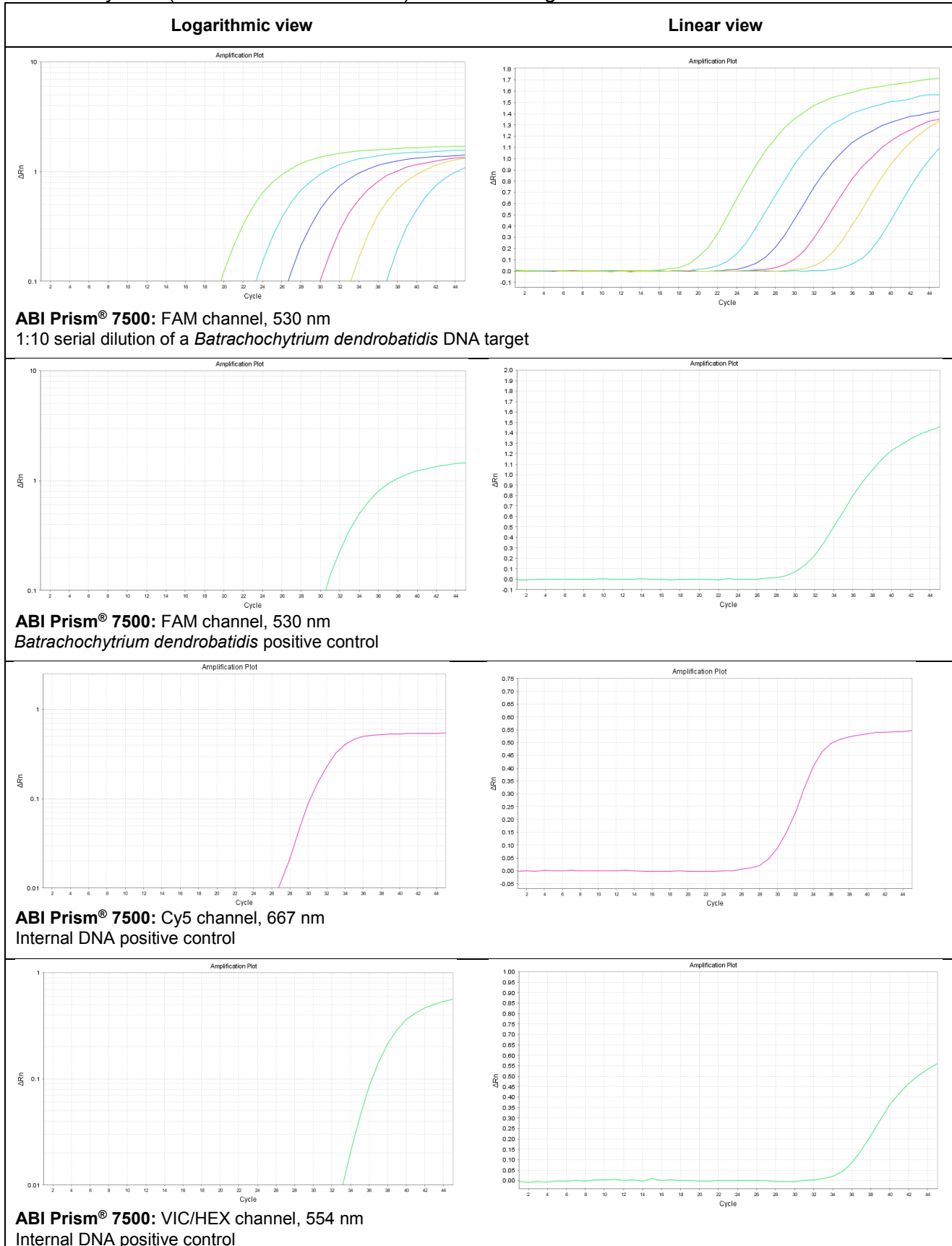


Figure 2 Performance of MycoReal Kit *Batrachochytrium dendrobatidis*