BactoReal® Kit
Leptospira spp. (16S rDNA)

Manual

For use with the

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

For veterinary use only

REF	**DVEB00513**	Σ	**100**
REF	**DVEB00553**	Σ	**50**

ingenetix GmbH
Arsenalstraße 11
1030 Vienna, Austria
T +43(0)1 36 1980 198
F +43(0)1 36 1980 199
office@ingenetix.com
www.ingenetix.com

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1. Product description

BactoReal® Kit *Leptospira* spp. (16S rDNA) is a real-time PCR assay for detection of DNA of pathogenic and intermediately pathogenic *Leptospira* species. This test was developed and validated for the ABI PRISM® 7500 (Fast) instrument (Life Technologies), LightCycler® 480 (Roche) and Mx3005P® (Agilent), but is also suitable for other real-time PCR instruments. The test facilitates the rapid and sensitive detection of DNA of pathogenic and intermediately pathogenic *Leptospira* species from samples purified from blood, urine or kidney tissue. *Leptospira* DNA can be recovered efficiently from sample material using the QIAamp DNA Mini Kit extraction methods, for example.

BactoReal® Kit *Leptospira* spp. (16S rDNA) detects the 16S rDNA gene of pathogenic and intermediately pathogenic *Leptospira* species. A probe-specific amplification-curve at 554 nm (VIC/HEX channel) indicates the amplification of *Leptospira* species specific DNA. An internal positive control system for detection in Cy5 channel (667 nm) 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

The spirochaetal *Leptospira* genus consists of pathogenic species (*L. interrogans, L. noguchii, L. weilii, L. kirschneri, L. alexanderi, L. borgpetersenii, L. santarosai, L. kmetyi, Leptospira* genomospecies 1), of intermediately pathogenic species (*L. inadai, L. fainei, L. broomii, L. licerasiae, L. wolffii*) and of non- pathogenic species (*L. biflexa, L. meyeri, L. wolbachii, Leptospira* genomospecies 3, 4 and 5). Members of *Leptospira* can also be grouped into serovars. Currently over 200 serovars are recognized in the genus *Leptospira* and a few serovars are found in more than one species of *Leptospira*. Non-pathogenic species are saprophytes and can grow outside the host animal, while pathogenic *Leptospira* species cause leptospirosis. They affect many mammalian species, including humans. Animals may become unapparent carriers, and shedding of leptospires, primarily in the urine, serves as a source of infection for other animals and humans. The clinical signs associated with leptospirosis are variable and depend on the infecting serovar and the susceptibility of the animal. Leptospires are present in the blood during the first 5 to 10 days after onset of the disease.

References:

- Levett, P.N. 2001. Leptospirosis. Clin. Microbiol. Rev. 14:296–326.
- Chen J, Bergevin J, Kiss R, Walker G, Battistoni T, Lufburrow P, Lam H, Vinther A. 2012. Case Study: A novel bacterial contamination in cell culture production - *Leptospira licerasiae*. PDA J Pharm Sci Technol. 66:580-91.

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. Contents of the Kit

Labelling	Content	Amount		Storage
		DVEB00513	DVEB00553	
Leptospira spp. 16S rDNA Assay Mix (purple cap)	Primer and probe (VIC/HEX) for detection of *Leptospira*	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
Leptospira spp. 16S rDNA 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 *Leptospira* spp. (16S rDNA) are stable until the expiry date stated on the label. Repeated thawing and freezing should be avoided. Please protect kit components from light.

5. 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 VIC/HEX and Cy5 channel
- Appropriate 96 well reaction plates or reaction tubes with corresponding (optical) closing material

6. 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 *Leptospira* 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.

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
	Leptospira spp. 16S rDNA Assay Mix	1.0 µl
	CR-3 Assay Mix	1.0 µl
	Total volume Master Mix	**15.0 µl**
Preparation of PCR	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 use 1 µl of the *Leptospira* spp. (16S rDNA) 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: VIC/HEX-TAMRA for detection of *Leptospira*
Cy5-NONE (CR-3 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 (VIC/HEX channel) Leptospira 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 *Leptospira* spp. (16S rDNA) please select fluorescence display options VIC/HEX channel for the *Leptospira* target and Cy5 channel 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.

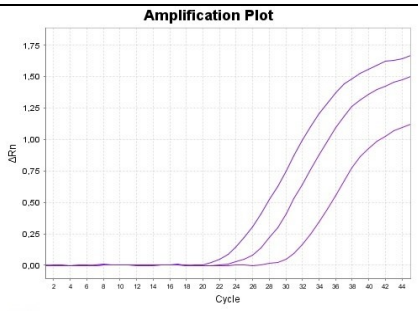
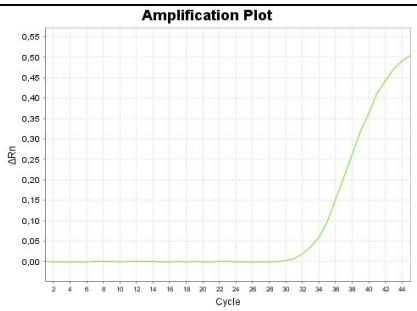
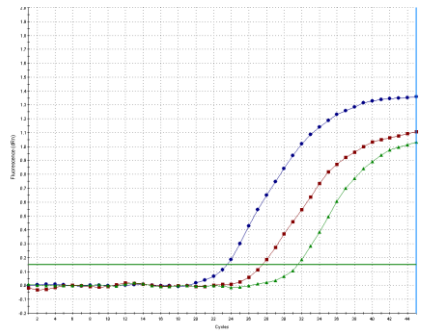
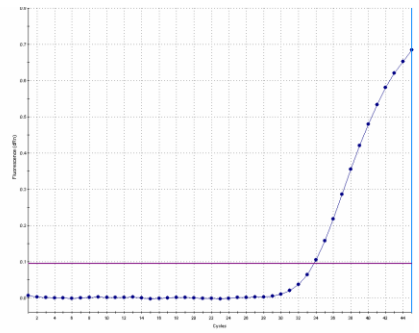
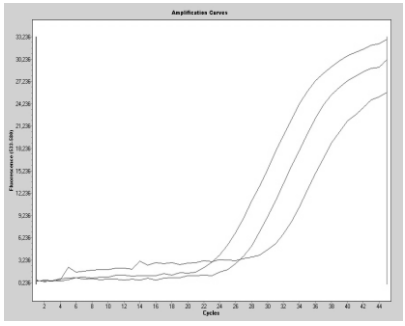
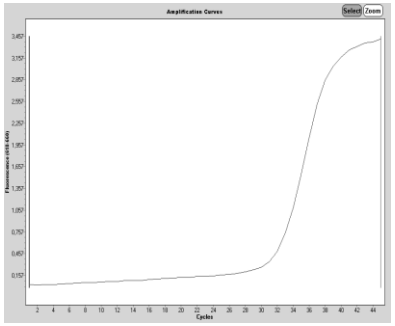
Once the analysis is completed, the following results are possible:

1. Signal in VIC/HEX channel:
→ DNA of pathogenic or intermediately pathogenic *Leptospira* was amplified. The sample has to be interpreted as positive.
Leptospira DNA can lead to a reduced or absent fluorescence signal of the internal positive control (competition of PCR).

2. No signal in VIC/HEX channel:
→ No DNA of pathogenic or intermediately pathogenic *Leptospira* is detectable in the sample. The sample has to be interpreted as negative.
An inhibition of PCR cannot be excluded.

2a. No signal in VIC/HEX channel but signal of the internal positive control:
→ No DNA of pathogenic or intermediately pathogenic *Leptospira* 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 VIC/HEX 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 ***Leptospira* spp.**	Detection of internal positive control **CR-3**
	
ABI Prism® 7500: VIC channel, 554 nm 1:10 serial dilution of *Leptospira* DNA	**ABI Prism® 7500:** Cy5 channel, 667 nm Internal positive control
	
Mx3005P®: HEX channel 1:10 serial dilution of *Leptospira* DNA	**Mx3005P®:** CY5 channel Internal positive control
	
LightCycler® 480: VIC / HEX / Yellow555 channel 1:10 serial dilution of *Leptospira* DNA	**LightCycler® 480:** Cy5 channel Internal positive control

9. Troubleshooting

1. No *Leptospira* 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 *Leptospira* 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. *Leptospira* 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. *Leptospira* 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 *Leptospira* spp. (16S rDNA) 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 10 target copies/PCR reaction. The limit of detection (LoD95 = smallest number of copies of target RNA which can be detected in 95% of cases) is 20 target copies/reaction and 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 pathogenic and intermediately pathogenic *Leptospira* strains.
BactoReal® Kit *Leptospira* spp. (16S rDNA) was tested with 13 pathogenic, 1 intermediately pathogenic and 3 non-pathogenic *Leptospira* serovars as well as 2 *Leptonema* species. All species were correctly analyzed (see Table 1).

Table 1:

Tested isolates	PCR results
Leptospira interrogans serovar *icterohaemorrhagiae*	Positive
Leptospira interrogans serovar *autumnalis*	Positive
Leptospira interrogans serovar *bataviae*	Positive
Leptospira interrogans serovar *bratislava*	Positive
Leptospira interrogans serovar *canicola*	Positive
Leptospira interrogans serovar *australis*	Positive
Leptospira interrogans serovar *pomona*	Positive
Leptospira interrogans serovar *saxkoebing*	Positive
Leptospira interrogans serovar *wolfii*	Positive
Leptospira interrogans serovar *hebdomadis*	Positive
Leptospira kirschneri serovar *grippotyphosa*	Positive
Leptospira borgpetersenii serovar *sejroe*	Positive
Leptospira borgpetersenii serovar *hardjo*	Positive
Leptospira licerasiae	Positive
Leptospira biflexa serovar *patoc*	Negative
Leptospira biflexa serovar *andamana*	Negative
Leptospira genomospecies 3 serovar Holland	Negative
Leptonema illini	Negative
Leptonema dimbowitza	Negative

11. Annex – symbols

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