BactoReal® Kit *Streptococcus* spp.

Manual

For use with the

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

For research only, not for diagnostic use

REF **DVEB03811, DVEB03813** Σ **100**

REF **DVEB03851, DVEB03853** Σ **50**

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1. Product description

BactoReal® Kit *Streptococcus* spp. is a real-time PCR assay for detection of DNA of species of the genus *Streptococcus*. This test was developed and validated for the ABI PRISM® 7500 (Fast) instrument (Applied Biosystems), LightCycler® 480 (Roche) and Mx3005P® (Agilent), but is also suitable for other real-time PCR instruments. This test allows the rapid and sensitive detection of DNA of *Streptococcus* spp. from samples purified from biopsies, blood, swabs, milk, etc. Extraction is recommended with InstaGene Matrix (Bio-Rad) or with QIAamp Viral RNA Mini Kit (Qiagen) to avoid contamination with streptococci.

BactoReal® Kit *Streptococcus* spp. detects the 23S rRNA gene of *Streptococcus* spp. A probe-specific amplification-curve at 530 nm (FAM channel) indicates the amplification of *Streptococcus* spp. specific DNA.
An internal positive control system for detection in VIC/HEX channel, (554 nm, order no. DVEB03811 or DVEB03851) or Cy5 channel (667 nm; order no. DVEB03813 or DVEB03853) 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® 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

Streptococcus is a genus of Gram-positive bacteria belonging to the *Firmicutes* and the lactic acid bacteria group. There are currently over 50 species known in this genus. Many streptococcal species are nonpathogenic and form part of the commensal animal or human microbiome of the mouth, skin, intestine, and upper respiratory tract. Certain *Streptococcus* species are responsible for streptococcal pharyngitis, pink eye, meningitis, bacterial pneumonia, endocarditis, erysipelas, necrotizing fasciitis and bovine mastitis.

References:
Wyder AB, Boss R, Naskova J, Kaufmann T, Steiner A, Graber HU. 2011. *Streptococcus* spp. and related bacteria: their identification and their pathogenic potential for chronic mastitis - a molecular approach. Res. Vet. Sci. 91:349-57.

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 *Streptococcus* spp. 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.

5. Contents of the Kit

5.1. BactoReal® Kit *Streptococcus* spp. order no. DVEB03811 or DVEB03851

Labelling	Content	Amount		Storage
		DVEB03811	DVEB03851	
Streptococcus spp. Assay Mix (green cap)	Primer and probe (FAM) for detection of *Streptococcus*	2 x 50 µl	1 x 50 µl	-20°C
CR-1 Assay Mix (yellow cap)	Primer and probe (VIC/HEX) for detection of IPC	2 x 50 µl	1 x 50 µl	-20°C
Streptococcus spp. Positive Control (red cap)*	Control-DNA (approx. 1,000 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

*Optional: a 1:10 dilution of the positive control can be used and defined as second standard value (approx. 100 target copies/µl).
#DNA Reaction Mix contains uracil-N glycosylase (UNG)

5.2. BactoReal® Kit *Streptococcus* spp. order no. DVEB03813 or DVEB03853

Labelling	Content	Amount		Storage
		DVEB03813	DVEB03853	
Streptococcus spp. Assay Mix (green cap)	Primer and probe (FAM) for detection of *Streptococcus*	2 x 50 µl	1 x 50 µl	-20°C
CR-3 Assay Mix (yellow cap)	Primer and probe (Cy5) for detection of IPC	2 x 50 µl	1 x 50 µl	-20°C
Streptococcus spp. Positive Control (red cap)*	Control-DNA (approx. 1,000 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

*Optional: a 1:10 dilution of the positive control can be used and defined as second standard value (approx. 100 target copies/µl).
#DNA Reaction Mix contains uracil-N glycosylase (UNG)

The components of BactoReal® Kit *Streptococcus* spp. 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
- Disposable powder-free gloves
- Pipettes (adjustable)
- Sterile pipette tips with filters
- Vortex mixer
- Desktop centrifuge with rotor for 2 ml reaction tubes

6.1. For ABI PRISM® 7500 Fast instrument (Applied Biosystems)

- *Either:* MicroAmp Fast Reaction Tubes (125 strips; 8 tubes/strip) (order no. 4358293) + MicroAmp Optical 8-Cap Strip (300 strips; 8 Tubes/Strip) (order no. 4323032)
- *Or:* MicroAmp Fast Optical 96-well reaction plate with barcode (0.1 ml) (20 plates) (order no. 4346906) + MicroAmp Optical Adhesive Film (100 pieces) (order no. 4311971)
- 7500 Fast Precision Plate Holder for MicroAmp Tube Strips

6.2. For ABI PRISM® 7500 instrument (Applied Biosystems)

- *Either:* ABI PRISM™ Optical Tubes (8 Tubes/ Strip) (125 strips; 8 tubes/strip) (order no. 4316567) + MicroAmp Optical 8-Cap Strip (300 strips; 8 Tubes/Strip) (order no. 4323032)
- *Or:* 96-Well Optical Reaction Plate with barcode (20 plates) (order no. 4306737) + Optical Adhesive Cover Starter Kit (20 pieces) (order no. 4313663)

6.3. For Mx3005P® QPCR System (Agilent)

- *Either:* 96-well PCR plates, 0.2 ml, non-skirted (order no. 401333)
  Or: 8 x strip tubes, 0.2 ml (order nr. 401428) and 8 x optical strip caps (order no. 401425)

6.4. For LightCycler® 480 (Roche)

- LightCycler® 480 Multiwell Plate 96, white with sealing foils (order no. 04729692001)

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
	Streptococcus spp. Assay Mix	1.0 µl
	CR 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**

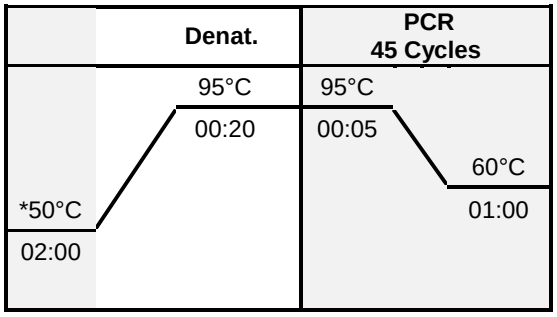
*1-8 µl of the sample can be used. When using an amount other than 5 µl of the sample, the amount of H_2O has to be changed accordingly.

Positive Control: As positive control use 1 µl of the *Streptococcus* spp. Positive Control + 4 µl H_2O.

7.2. Programming of the temperature profile

Please find further information on programming the real-time PCR instrument in the respective operator's manual.

- **Sample Volume:** 20 µl
- **Temperature Profile:**



***Note:** Optionally 50°C 15 min (reverse transcription step) can be used. These instrument parameters can then be used for all ViroReal®, BactoReal® and ParoReal kits (ingenetix) on all PCR instruments.

7.2.1. ABI PRISM® 7500 (Fast) instrument

Instrument parameter for *Absolute Quantification*:

- **Thermal Cycler Conditions (for ABI Real-Time PCR Systems):** without "fast cycling" parameter
- **Detectors:** FAM-TAMRA for detection of *Streptococcus* spp.
  Cy5-NONE for detection of internal positive control (CR-3 Assay Mix)
  or
  VIC-TAMRA for detection of internal positive control (CR-1 Assay Mix)
- **Passive Reference:** ROX

7.2.2. Mx3005P® instrument

- **Instrument parameters for *Quantitative PCR*:**
  Collect fluorescence data
  - **Select dyes:** FAM and Cy5 (CR-3 Assay Mix) **or** HEX (CR-1 Assay Mix)
  - **Select passive reference dye**: ROX
  - **Filter set gain settings:** ROX x2, Cy5 or HEX x4, FAM x8

7.2.3. LightCycler® 480 instrument

	Program name	Acquisition mode	Cycle	Analysis mode
1: Program	50°C, 2 min	none	1 cycle	None
2: Program	95°C, 20 sec	none	1 cycle	None
3: Program	95°C, 5 sec 60°C, 1 min	none single	45 cycles	Quantification
4: Program	40°C, 10 sec	none	1 cycle	None

Detection format:
Name: 2 Color Hydrolysis Probe
Integration time mode: Dynamic

Filter combinations:

Active	Name	Melt factor	Quant factor	Max integration time
Yes	FAM (465-510)	1	10	2 second(s)
Yes	Cy 5 / Cy 5.5 (618-660)	1	10	2 second(s)

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) *Streptococcus* target	Ct/Cp IPC target	Interpretation
Negative control	Negative / weak positive*	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 (recommended)	Negative / weak positive*	36.0 ± 2	Valid
Negative sample	Negative / weak positive*	36.0 ± 2	Valid
Positive sample	Positive**	Positive / Negative	Valid

*Streptococci can be found ubiquitously in the environment. Therefore, contamination with streptococcal DNA can lead to false-positive results. Contamination might happen during sample taking, DNA extraction and preparation of the PCR-reaction or might be due to contaminated reagents. Ct/Cp values >35 might result from the presence of low concentration of contaminating streptococcal DNA.
**A *Streptococcus*-specific amplification curve of the sample has to be interpreted in context of the Ct/Cp-values of the negative controls: The Ct/Cp value of a positive sample has to be at least 3 Ct/Cp values lower than that of the negative controls. High concentrations of some non-*Streptococcus* species might lead to weak cross reaction.

For analysis of PCR data please proceed as follows:
For analysis of PCR results gained with BactoReal® Kit *Streptococcus* spp. please select fluorescence display options FAM channel for the *Streptococcus* target and VIC/HEX channel (order no. DVEB03811, DVEB03851) or Cy5 channel (order no. DVEB03813, DVEB03853) 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.

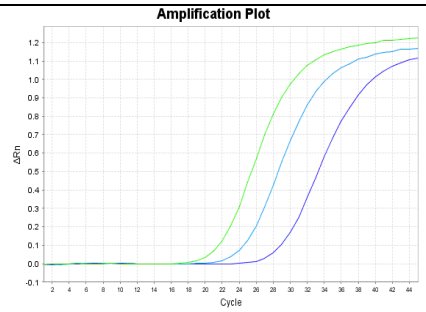
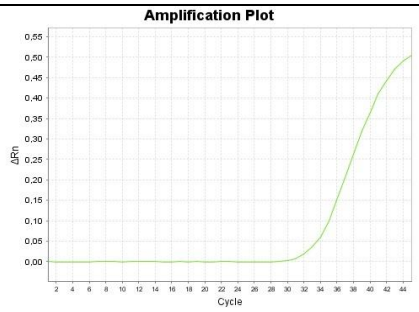
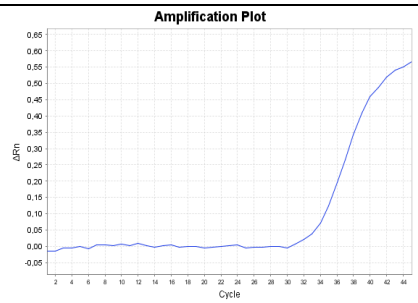
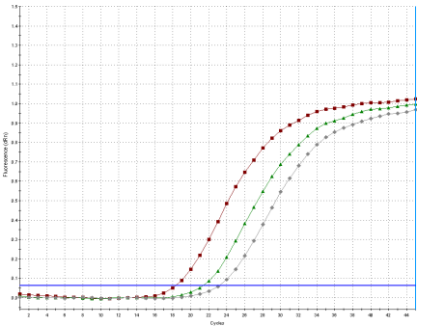
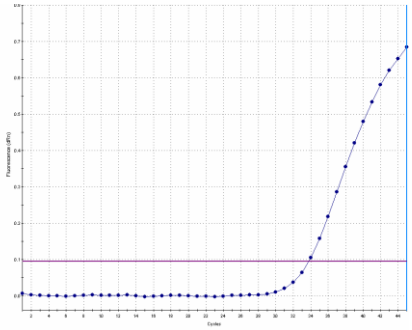
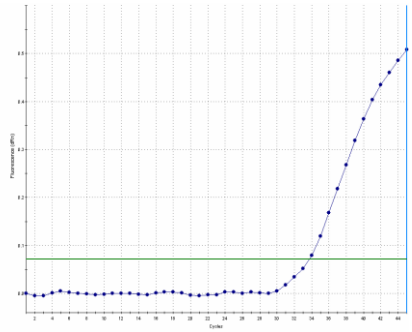
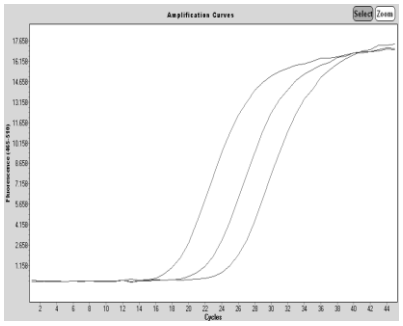
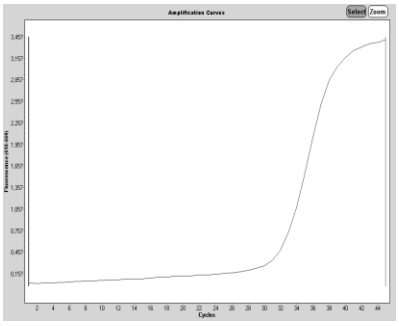
Once the analysis is completed, the following results are possible:

1. Signal in FAM channel:
→ DNA of *Streptococcus* was amplified. The sample has to be interpreted as positive (see also criteria for valid interpretation above).
DNA of *Streptococcus* can lead to a reduced or absent fluorescence signal of the internal positive control (competition of PCR).

2. No signal in FAM channel:
→ No DNA of *Streptococcus* 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 DNA of *Streptococcus* 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 ***Streptococcus* spp.**	Detection of internal positive control **CR-3**	Detection of internal positive control **CR-1**
		
ABI Prism® 7500: FAM channel, 530 nm 1:10 serial dilution of *Streptococcus* 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 serial dilution of *Streptococcus* DNA	**Mx3005P®:** CY5 channel Internal positive control	**Mx3005P®:** HEX channel Internal positive control
		
LightCycler® 480: FAM channel 1:10 serial dilution of *Streptococcus* DNA	**LightCycler® 480:** Cy5 channel Internal positive control	

9. Troubleshooting

1. No *Streptococcus* 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 *Streptococcus* 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. *Streptococcus* specific signal with the negative control: see also 8. Interpretation of PCR-data

- 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. *Streptococcus* specific signal with the negative control of DNA-extraction: see also 8. Interpretation of PCR-data

- 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 *Streptococcus* spp. was evaluated with the ABI PRISM® 7500 (Fast) instrument (Applied Biosystems), with the LightCycler® 480 (Roche) and the Mx3005P® (Agilent). For further validation data please contact ingenetix.

10.1. Analytical sensitivity

The analytical sensitivity is 50-100 copies per PCR, as in negative samples the presence of contaminating streptococcal DNA in the extraction or amplification reagents results in weak positive amplification.

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 *Streptococcus* species. High concentrations of non-*Streptococcus* species might lead to weak cross reaction.

11. Annex – symbols

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