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V For *in vitro* Veterinary Diagnostics only.

Kylt® Mycoplasma bovis

Real-Time PCR Detection Kit
for *Mycoplasma bovis*

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100 / 25 reactions
in vitro Diagnosticum for ruminants

A. General

- Kylt® Mycoplasma bovis Real-Time PCR Detection Kit is for detection of *Mycoplasma bovis* in samples from ruminants, such as swabs (e.g. from nose, trachea or lung, ear, joint and udder), tissues and organs, milk and material derived from cultural processes with the aforementioned samples.
- Kylt® Mycoplasma bovis comprises all reagents and controls for detection of bacterial DNA of *Mycoplasma bovis*. The qualitative testing of Kylt® Mycoplasma bovis is based on a duplex Real-Time PCR system: The target gene specific for *Mycoplasma bovis* as well as for the Internal Amplification Control are amplified in parallel by respective primer pairs in the subsequent Polymerase Chain Reaction (PCR) in one reaction vessel. Amplified target gene fragments are detected via fluorescently labeled probes during the PCR reaction in real-time (Real-Time PCR). The probes specific for detection of target genes of amplified *Mycoplasma bovis* and Internal Amplification Control are labeled with fluorescent dyes FAM and HEX, respectively, and their emitted fluorescence is separately optically measured by the Real-Time PCR thermal cycler. By means of two individual analyses in one reaction vessel per sample and the Negative Control and Positive Control per run the *Mycoplasma bovis* -specific status of a sample can be evaluated in the end. This way, results can be achieved within few hours after sample receipt.
- This kit was developed for use by trained laboratory personnel following standardized procedures. This Direction For Use must be followed strictly.

B. Reagents and Materials

- Kylt® Mycoplasma bovis Real-Time PCR Detection Kit contains the following reagents:

Reagent	Color code of lid	Quantity in kit with 100 / 25 reactions	Storage conditions
Reaction-Mix	violet	4 x / 1 x 500 µl	+2 °C to +8 °C
Positive Control	red	2 x / 1 x lyophilisate (final 20 µl each)	+2 °C to +8 °C lyophilized -18 °C to -20 °C rehydrated
Negative Control (Nuclease-free water)	blue	1 x 1 ml	+2 °C to +8 °C

- The kit and its components are stored at +2 °C to +8 °C. Alternatively, for long term storage the kit can be stored at -18 °C to -20 °C. Avoid repeated freezing and thawing of the kit or its components. If occasional processing of few samples is expected, you may prepare aliquots of the Reaction-Mix before storage at -18 °C to -20 °C. Reaction-Mix has to be stored dark, do not expose to direct sunlight!

- Before the first use the lyophilized Positive Control is rehydrated: 20 µl of the Negative Control (Nuclease-free water) are added per vial of Positive Control, briefly incubated at room temperature and mixed thoroughly by repeated vortexing. Storage of aliquots with 5 - 10 µl volume (depending on the expected number of Positive Control reactions per kit) at -18 °C to -20 °C is recommended.

- This kit can be used on all commercially available Real-Time PCR thermal cyclers that detect the emitted fluorescence of the fluorescent dyes FAM (emission 520 nm) and HEX (emission 550 nm).

- We recommend to exclusively using certified Nuclease-free materials and powder-free protective gloves. Pipette tips have to be changed between samples to avoid cross-contamination. Gloves have to be changed frequently, especially after spillage or other suspected contaminations.

- Apart from the disposables, the following devices are needed (not included in this kit):
  - DNA preparation kit (e.g. Kylt® RNA/DNA Purification or Kylt® DNA Extraction-Mix II)
  - Table top microcentrifuge
  - Vortex
  - Micropipettes volume range 1 - 1000 µl
  - Centrifuge for PCR tubes or plates
  - Real-Time PCR thermal cycler

C. Control Reactions

- The Positive Control included in this kit allows for control of the specificity and efficiency of the reagents and the reaction itself. The Negative Control included in the kit allows exclusion of contaminations. The test is only valid if both, Positive and Negative Controls, are used and verified for validity in every Real-Time PCR run.

- An Internal Amplification Control is included in the Reaction-Mix in defined copy number; it is co-amplified (channel HEX) in every single reaction to detect possible inhibitory effects of the DNA-Extract and to verify true negative results.

D. Protocol

- The overall protocol of this *Mycoplasma bovis*-analysis consists of the following steps:
  1. Sample preparation
  2. DNA extraction
  3. Real-Time PCR Setup and Amplification
  4. Data Analysis – Validity and Qualitative Result

1.Sample Preparation

- We recommend pooling of not more than five individual samples or samples of more than five individuals, respectively, per DNA preparation.
- Swabs are pooled in a sufficient volume of sterile buffer (e.g. Normal Saline or 0.1 x TE) and soaked for an adequate period of time. Then, the sample is washed out thoroughly by pulse-vortexing. The washed out supernatant is used for DNA preparation. Single small swabs may directly be immersed in lysis buffer.
- Tissue samples are thoroughly homogenized in sterile buffer (see above) and a suitable volume is administered for DNA preparation.
- Cultural material is directly processed with an appropriate DNA preparation protocol.

2.DNA Extraction

- DNA that was prepared with commercially available DNA preparation kits, such as the Kylt® RNA/DNA Purification or alternative kits is suitable for the application of Kylt® Mycoplasma bovis. For detailed information on the DNA preparation process, please refer to the Direction For Use or Standard Operating Procedure of the respective kit or in-house method, respectively.

3.Real-Time PCR Setup and Amplification

- Reaction-Mix and Negative Control should be vortexed and spun down before each use.
- Determine the number of needed PCR-reactions needed: number of samples + Positive Control and Negative Control.
- Pipette 18 µl of Reaction-Mix to each PCR reaction tube / each PCR-plate well (»cavity«). Keep exposure of Reaction-Mix to light as short as possible!
- Add 2 µl of Negative Control to corresponding cavity and seal.
- Add 2 µl of sample DNA-Extract to corresponding cavity and seal.
- To minimize risk of potential cross-contaminations, 2 µl of the Positive Control are added to the corresponding cavity after all previous samples and control reactions are set up. Before each use, briefly vortex and spin down the rehydrated Positive Control (see also chapter B “Reagents and Materials”).
- Avoid formation of bubbles when pipetting Reaction-Mix, samples and controls. It is recommended to always centrifuge cavities before the PCR run.

- Place the cavities in the Real-Time PCR thermal cycler and amplify using the following parameters:

Step	Temperature	Duration	
Activation Polymerase	95 °C	10 min	
Denaturation	95 °C	15 sec	42 cycles
Annealing & Extension	60 °C	1 min	
Fluorescence Detection	channels FAM and HEX		

- Please follow the specific instructions of your Real-Time PCR thermal cycler as recommended by the manufacturer.
- The amplification parameters allow the combination of this Kylt® Mycoplasma bovis Real-Time PCR with other Kylt® Real-Time PCRs for detection of bacteria. When combining Kylt® detection tests for different pathogens, make sure all necessary channels are used!

4. Data Analysis – Validity and Qualitative Result

General

- The amplification data can be processed automatically using the specific software tool of your Real-Time PCR thermal cycler. Alternatively, the threshold can be set manually considering the following directions: The threshold should cross the FAM-curve and the HEX-curve in the linear increase of their slope (log scaling of the y-axis). By setting the threshold, the crossing points with the HEX- and FAM-curves determine the respective cycle threshold (Ct), which is negatively correlated with the initial concentration of copies of the target genes in the Real-Time PCR reaction.
- Only curves with the typical exponential amplification, meaning the curve of the raw data shows a fl at baseline at the beginning, followed by a clear (exponential) slope in fluorescence and possibly reaching a plateau-phase (y-axis in logarithmic scaling), should be regarded as positive.
- The actual test analysis starts with the validity check of the entire Real-Time PCR run. Afterwards, by means of the Internal Control the validity of each sample reaction and its true test result can be verified according to the Ct-value of the Internal Control channel (HEX). Finally, the *Mycoplasma bovis* -specific status of each sample is analyzed (FAM).

Test Evaluation

- The test is only valid if the FAM-curve of the Negative Control is negative, if the HEX-curve of the Negative Control is positive and if the FAM-curve of the Positive Control is positive.

HEX-curve positive	yes	yes	no	no
FAM-curve positive	no	yes	yes	no
The sample is *Mycoplasma bovis*	**negative**	**positive**	**positive**	**inhibited**

- A sample is negative if its HEX-curve is positive, but its FAM-curve is negative.
- A **sample** is **positive** if its FAM-curve is positive, independent of the HEX-curve.
- A **sample** is **inhibited** if neither the FAM-curve nor the HEX-curve is positive.

- **Recommendation**: In the case of an inhibited sample the test should be repeated with a dilution of the DNA extract at 1:10 (9 volumes Negative Control + 1 volume DNA extract or eluted DNA). The Negative Control serves as the diluting agent. Preferably, the entire DNA preparation process is repeated.
- Convenient and reliable sample data entry, Real-Time PCR start, final qualitative analysis and documentation can be conducted with the Kylt® PCR Software package, please inquire.

Production:

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