Kylt® Haemophilus parasuis

Real-Time PCR Detection Kit for detection of Haemophilus parasuis

www.kylt.eu
A. Introduction

- Kylt® Haemophilus parasuis Real-Time PCR Detection Kit is for genus-specific detection of Haemophilus parasuis in samples from swine such as feces, swabs, tissues and organs and sampling material derived from cultural processes with the aforementioned samples.

- Kylt® Haemophilus parasuis comprises all reagents and controls for detection of bacterial DNA of Haemophilus parasuis. The qualitative testing of Kylt® Haemophilus parasuis is based on a duplex Real-Time PCR system: the target genes specific for Haemophilus parasuis 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 Haemophilus parasuis 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 Haemophilus parasuis-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® Haemophilus parasuis contains the following reagents:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Colour code of lid</th>
<th>Quantity in kit with 25 / 100 reactions</th>
<th>Storage conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reaction-Mix</td>
<td>violet</td>
<td>1 x / 4 x 500 µl</td>
<td>+2 °C to +8 °C</td>
</tr>
<tr>
<td>Positive Control</td>
<td>red</td>
<td>2 x / 4 x lyophilisate (final 20 µl each)</td>
<td>+2 °C to +8 °C lyophilized -18 °C to -20 °C rehydrated</td>
</tr>
<tr>
<td>Negative Control</td>
<td>blue</td>
<td>1 x 1 ml</td>
<td>+2 °C to +8 °C</td>
</tr>
</tbody>
</table>

- 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 its first application rehydrate both lyophilized Positive Control by adding 20 µl of Negative Control per vial, briefly incubate at room temperature and mix by repeated vortexing. Storage of aliquots with 5 µl to 10 µl (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 and HEX (emission: 520 nm and 550 nm).

- We recommend the exclusive use of certified Nuclease-free disposables as well as powder-free protective gloves. Please wear gloves during the entire experimental procedure. Gloves need to be changed frequently, especially after spillage or suspected contaminations. Pipette tips must be changed between each sample to avoid cross-contaminations.

- Apart from the disposables, the following devices are needed (not included in this kit):
  - DNA preparation kit / protocol (e.g. Kylt® RNA/DNA Purification)
  - Table top microcentrifuge
  - Vortex
  - Micropipettes covering volumes of 1 µl to 1000 µl
  - Centrifuge for PCR tubes or plates
  - Real-Time PCR thermal cycler
C. Control Reactions

- The **Negative Control** included in this kit provides the ability to exclude possible contaminations of the reagents. The **Positive Control** included in this kit provides the ability to monitor the specificity and efficiency of the reagents used, i.e. the performance of Real-Time PCR itself and of the Real-Time PCR thermal cycler, respectively. The sample testing is only valid if both, the Negative Control and the Positive Control, are included in every Real-Time PCR run and used to verify the validity of such run.

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

- If appropriate sampling is unsure we recommend to analyze in parallel with Real-Time (RT-)PCR specific for housekeeping genes of birds and swine, such as the Kytt® Host Cells Real-Time RT-PCR Detection Kit.

D. Protocol

- The overall protocol of the analysis consists of the following workflow:
  1. sample preparation and DNA purification
  2. reaction setup and amplification (Real-Time PCR)
  3. data analysis – validity and qualitative result

1. Sample Preparation and DNA purification

- For sample preparation and DNA purification use a commercially available DNA purification kit (such as Kytt® RNA/DNA Purification kit) or, if available, an appropriate in-house method. Please carry out the DNA purification according to the manufacturer’s instructions of the DNA purification kit or according to the standard operating procedure of the respective in-house method.

2. Reaction Setup and Amplification

- Before each use, briefly vortex and spin down the Reaction-Mix and Negative Control.

- Determine the number of needed PCR-reactions: 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 the prepared DNA (DNA extract or eluted DNA) of sample to corresponding cavity and seal. Solely use clear supernatant of DNA extract, avoid transferring debris.

- Once all sample cavities are sealed, 2 µl of the **Positive Control** is added to the corresponding cavity and sealed. The Positive Control is vortexed and spun down before each use.
- Avoid formation of bubbles when pipetting Reaction-Mix, samples and controls. It is recommended to consistently spin down cavities before the PCR run.

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

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activation of Polymerase</td>
<td>95 °C</td>
<td>10 min</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95 °C</td>
<td>15 sec</td>
</tr>
<tr>
<td>Annealing &amp; Extension</td>
<td>60 °C</td>
<td>60 sec</td>
</tr>
<tr>
<td>Fluorescence Detection</td>
<td>Channels FAM and HEX</td>
<td></td>
</tr>
</tbody>
</table>

- Please follow the specified instructions of your Real-Time PCR thermal cycler as recommended by the manufacturer.

- The amplification parameters above allow for combination of this Kylt® Haemophilus parasuis Real-Time PCR with other Kylt® Real-Time PCRs, e.g. for detection of Salmonella species. In the event of a combined Real-Time PCR run, make sure all necessary channels are used.

3. Data Analysis – Validity and Qualitative Result

General

- The data of the amplification reactions can automatically be processed using specific software for the Real-Time PCR thermal cycler. Alternatively a threshold can be set manually. The threshold should cross the HEX-curve of the Negative Control and the FAM-curve of the Positive Control, respectively, in the linear area of its slope. By setting the thresholds, 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 flat 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 considered as positive.

- The actual test analysis starts with the validity check of the entire Real-Time PCR run. Afterwards, by means of the Internal Amplification 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 Haemophilus parasuis-specific status of each sample is analyzed (channels FAM).
Test Evaluation

- The Real-Time PCR test run 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.

<table>
<thead>
<tr>
<th>HEX-curve is positive</th>
<th>yes</th>
<th>yes / no</th>
<th>no</th>
</tr>
</thead>
<tbody>
<tr>
<td>FAM-curve is positive</td>
<td>no</td>
<td>yes</td>
<td>no</td>
</tr>
</tbody>
</table>

The sample is **Haemophilus parasuis**

- **Negative for Haemophilus parasuis** if its FAM-curve is negative and the HEX-curve is positive.
- **Positive for Haemophilus parasuis** if its FAM-curve is positive, independent of the HEX-curve.
- **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 eluted DNA). The Negative Control serves as the diluting agent. Alternatively, the original sample or the DNA elution can be utilized for DNA purification using appropriate alternative procedures, such as repetition of purification with less starting material or purification with Kylt® RNA/DNA Purification kit.

- Convenient and reliable sample data input, start of the Real-Time PCR run as well as final qualitative test result analysis and documentation can be managed by the Kylt® Software package. Please feel free to contact us.

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