Kylt® Haemophilus parasuis

Real-Time PCR Detection

www.kylt.eu
A. General

- Kylt® HPS products are intended for the specific detection of bacterial DNA of *Haemophilus parasuis*. The products are suitable for the analysis of samples from swine, such as tissues and organs (e.g. lung, joints, heart), swabs of the aforementioned samples, and sampling material derived from cultural processes with the aforementioned samples.

- The qualitative testing with Kylt® HPS products is based on a duplex Real-Time PCR: In one reaction setting, the target genes for *Haemophilus parasuis* as well as for the Internal Control are amplified in parallel by respective primer pairs in the Polymerase Chain Reaction (PCR). 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 amplified *Haemophilus parasuis* and the Internal Control target genes 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 both 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 a few hours after sample receipt.

- These products were developed for use by trained laboratory personnel following standardized procedures. This Direction For Use must be followed strictly.
B. Reagents and Materials

- The following Kylt® HPS products are available and comprise the following reagents:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Colour of Lid</th>
<th>Quantity in Kit with 100 / 25 Reactions</th>
<th>Store at</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Article No 31372 / 31373</td>
<td>Article No 31553 / 31554</td>
</tr>
<tr>
<td>Reaction-Mix</td>
<td>orange</td>
<td>4 x / 1 x 450 µl</td>
<td>≤ -18 °C</td>
</tr>
<tr>
<td>Detection-Mix</td>
<td>yellow</td>
<td>-/-</td>
<td>≤ -18 °C</td>
</tr>
<tr>
<td>Positive Control</td>
<td>red</td>
<td>4 x / 2 x lyophilizate (final 50 µl each)</td>
<td>≤ -18 °C</td>
</tr>
<tr>
<td>Negative Control</td>
<td>blue</td>
<td>1 x 1 ml</td>
<td>≤ -18 °C</td>
</tr>
</tbody>
</table>

- After receipt, the components are immediately stored at ≤ -18 °C. Avoid repeated freezing and thawing of all the reagents and keep them thawed as short as possible. If occasional processing of few samples only is expected you may prepare appropriate aliquots of reagents before storage at ≤ -18 °C. Prepare aliquots in such a way that freeze-thaw-cycles are reduced to a maximum of three. The Negative Control can alternatively be stored at +2°C to +8°C.

- The components are to be used within the indicated shelf life (see label on the outer packing). The components of different batches may not be mixed.

- Before its first use, rehydrate the Positive Control: add 50 µl of Negative Control per vial, briefly incubate at room temperature and mix thoroughly by repeated vortexing. It is recommended to generate aliquots of suitable volumes and store them at ≤ -18 °C.

- The Reaction-Mix needs to be stored protected from abundant light. Do not expose to direct (sun) light.

- The Detection-Mix needs to be stored protected from abundant light. Do not expose to direct (sun) light. Before first use, rehydrate the lyophilized Detection-Mix: add 150 µl of the Negative Control per vial of Detection-Mix, briefly incubate at room temperature and mix by pulse-vortexing. Generate aliquots of suitable volumes and store them at ≤ -18 °C.

C. Equipment and Reagents not included

- This detection method 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 and 550 nm, respectively). Note that default normalization option against ROX (e.g. using ABI cyclers) must be deactivated.

- Apart from the disposables, the following further devices are needed and are not included in the Kylt® HPS products:
  - 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

- For Kylt® products supplied with Detection-Mix only, at least 2x concentrated qPCR-Mix is needed (e.g. BCD 2x qPCR-Mix).
Accessory Kylt® products: see chapter F “Related and Accessory Products”.

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.

D. Control Reactions

- The **Positive Control** allows for control of the specificity and efficiency of the reagents and the reaction itself, including the performance of the Real-Time PCR and of the Real-Time PCR thermal cycler.
- The **Negative Control** allows for exclusion of contaminations. The sample testing is only valid if both, Positive and Negative Controls, are used and verified for validity in every Real-Time PCR run.
- The **Internal Amplification Control** is included in the Reaction-Mix or Detection-Mix, depending on the product used, in a defined copy number; it is co-amplified (channel HEX) with every single reaction to detect possible inhibitory effects of the DNA preparation on the Real-Time PCR itself and thus to verify true-negative results.
- If appropriate sampling is unsure we recommend to analyze the samples in parallel with Kylt® Host Cells Real-Time RT-PCR Detection for presence of amplifiable nucleic acids derived from host cell material, see chapter F “Related and Accessory Products”.

E. Protocol *(see also „Protocol At A Glance“ at the end of this Direction For Use)*

The overall protocol of the analysis consists of the following main workflow:

1. Sample Preparation
2. DNA Preparation
3. Reaction Setup and Amplification (Real-Time PCR)
4. Data Analysis – Validity and Qualitative Result

We recommend proceeding through the protocol without interruption to avoid potential degradation of the processed samples and reagents. If necessary, you may store the final DNA preparation at ≤ -18 °C until further processing. Avoid repeated freezing and thawing of the DNA preparations.

1. Sample Preparation

- We recommend pooling of at most five samples or samples from five individuals, respectively, per DNA preparation.
- Pool **swabs** in a sufficient volume of sterile buffer (e.g. 1 ml of Normal Saline or 0.1 x TE), let the swabs soak for an adequate period of time and finally wash out the swabs by thorough pulse-vortexing. The washed out supernatant is used for DNA preparation. Small swabs may directly be immersed lysis buffer, if applicable.
- **Tissue and organ** samples are homogenized thoroughly in sterile buffer (see above) and a suitable volume is used for the DNA preparation.
- Material derived from cultural processes, i.e. **colony material**, is directly transferred into respective tubes for Kylt® DNA Extraction (please refer to 2 “DNA Preparation”), such as conical screw cap tube; therefore a little amount of a single colony is picked with a sterile loop wire or sterile pipette tip and transferred to the tube.
2. DNA Preparation

- All kinds of sample matrices, including pure isolates, swabs, tissues and organs may be processed with appropriate DNA preparation kits, such as Kylt® RNA/DNA Purification (please refer to chapter F “Related Products”) or appropriate in-house methods.

- For detailed information on the DNA preparation process, please refer to the Direction For Use or Standard Operating Procedure of the specific kit or in-house method, respectively.

3. Reaction Setup and Amplification (Real-Time PCR)

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

- To determine the total number of reactions needed, count the number of samples and add two more for the Negative Control and the Positive Control.

- In case of products supplied with Reaction-Mix this is ready-to-use, add 16 μl to each of the PCR tubes or plate wells (“cavities”).

- In case of products supplied with Detection-Mix:
  - Prepare the Master-Mix using the components listed below. It is strongly recommended to prepare a Master-Mix, consisting of qPCR-Mix (e.g. BCD 2x qPCR-Mix) and Detection-Mix, at least per PCR setup.
  - When calculating volumes for preparation of the Master-Mix, consider the concentration (e.g. “2x”) of the qPCR-Mix used. For more information about the qPCR-Mix please refer to the Direction For Use of the respective manufacturer.
  - Alternatively, when using the recommended BCD 2x qPCR Mix a larger volume of a ready to use working Master-Mix can be prepared and stored at ≤ -18 °C for convenient use over a longer period of time up to the expiry date given on the label. In case of frozen storage the working Master-Mix should be aliquoted in such a way that freeze-thaw-cycles are reduced to a maximum of three.
  - Vortex, spin down and add 16 μl of the finalized Master-Mix to each of the PCR tubes or plate wells (“cavities”).

<table>
<thead>
<tr>
<th>Example Calculation for 2x concentrated qPCR-Mix:</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent</td>
<td>per Reaction</td>
</tr>
<tr>
<td>qPCR-Mix (e.g. BCD 2x qPCR-Mix)</td>
<td>10 µl</td>
</tr>
<tr>
<td>Detection-Mix</td>
<td>6 µl</td>
</tr>
<tr>
<td><strong>Total Master-Mix</strong></td>
<td><strong>16 µl</strong></td>
</tr>
<tr>
<td>DNA (Negative Control / sample DNA / Positive Control)</td>
<td>4.0 µl</td>
</tr>
<tr>
<td><strong>Total Reaction</strong></td>
<td><strong>20.0 µl</strong></td>
</tr>
</tbody>
</table>

- Keep exposure of the Reaction-Mix, Detection-Mix and prepared Master-Mix to (sun)light as short as possible and return it back to appropriate storage temperature right after application. Avoid the formation of bubbles when pipetting the Master-Mix, samples and controls.

- Add 4 µl of the Negative Control to the corresponding cavity and seal it individually, if possible.

- Add 4 µl of each DNA preparation to the corresponding cavities and seal them individually, if possible.
To minimize risk of potential cross-contaminations, 4 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").

If not already done, finally seal the cavities. It is recommended to briefly spin them down before the start of the Real-Time PCR run.

Place the cavities in the Real-Time PCR thermal cycler and run the test with Kylt® Profile II as given below.

In case Kylt® products supplied with Detection-Mix are used in combination with BCD 2x qPCR Mix, preferably the given Kylt® Profile II is applied. For other qPCR Mixes, please refer to the respective Direction For Use, check for appropriateness and adapt duration or temperature of individual steps, if necessary.

Kylt® Profile II

<table>
<thead>
<tr>
<th>Step No</th>
<th>Description</th>
<th>Temperature</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Activation of Polymerase</td>
<td>95 °C</td>
<td>10 min</td>
</tr>
<tr>
<td>2</td>
<td>Denaturation</td>
<td>95 °C</td>
<td>15 sec</td>
</tr>
<tr>
<td>3</td>
<td>Annealing &amp; Extension</td>
<td>60 °C</td>
<td>1 min</td>
</tr>
<tr>
<td>4</td>
<td>Fluorescence Detection channels</td>
<td>FAM and HEX</td>
<td>42 cycles</td>
</tr>
</tbody>
</table>

Kylt® Profile II allows for combined run of this and most other Kylt® qPCR detection methods.

Alternatively, the Kylt® Profile I given below can be applied. Kylt® Profile I allows for combined run of this and most other Kylt® qPCR detection methods as well as Kylt® RT-qPCR detection products that need Reverse Transcription, such as those for detection of viral RNA.

In case of Kylt® products supplied with Detection-Mix please refer to the according Direction For Use of the qPCR-Mix used to check if using combined Kylt® Profile I is feasible.

Kylt® Profile I

<table>
<thead>
<tr>
<th>Step No</th>
<th>Description</th>
<th>Temperature</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Reverse Transcription</td>
<td>50 °C</td>
<td>10 min</td>
</tr>
<tr>
<td>2</td>
<td>Activation of Polymerase</td>
<td>95 °C</td>
<td>1 min</td>
</tr>
<tr>
<td>3</td>
<td>Denaturation</td>
<td>95 °C</td>
<td>10 sec</td>
</tr>
<tr>
<td>4</td>
<td>Annealing &amp; Extension</td>
<td>60 °C</td>
<td>1 min</td>
</tr>
<tr>
<td>5</td>
<td>Fluorescence Detection</td>
<td>channels FAM and HEX</td>
<td>42 cycles</td>
</tr>
</tbody>
</table>

In the event of a combined Real-Time (RT-)PCR run, make sure all necessary channels are detected.

Please follow the specified instructions of your Real-Time PCR thermal cycler as recommended by the manufacturer.
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 flat baseline at the beginning, followed by a clear (exponential) slope in fluorescence and possibly reaching a plateau-phase (y-axis in log 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 *Haemophilus parasuis*-specific status of each sample is analyzed (FAM).

Test Evaluation

- The **Real-Time PCR test run** is only **valid** if the FAM-curve of the Negative Control is negative, the HEX-curve of the Negative Control is positive and the FAM-curve of the Positive Control is positive. For a valid test the FAM-Ct-value of the Positive Control has to be > 15 and ≤ 35 and the HEX-Ct-value of the Negative Control has to be ≤ 40.

<table>
<thead>
<tr>
<th>Target</th>
<th>Channel</th>
<th>Signal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Internal Control</td>
<td>HEX</td>
<td>positive / negative</td>
</tr>
<tr>
<td><em>Haemophilus parasuis</em></td>
<td>FAM</td>
<td>negative</td>
</tr>
</tbody>
</table>

**The sample is *Haemophilus parasuis*** | negative | positive | inhibited

- A **sample** is **negative for *Haemophilus parasuis*** if its HEX-curve is positive (Ct ≤ 40), but its FAM-curve is negative.

- A **sample** is **positive for *Haemophilus parasuis*** if its FAM-curve is positive (Ct ≤ 42), independent of the HEX-curve.

- A **sample** is **inhibited** if neither the FAM-curve nor the HEX-curve are positive.

**Recommendation:** In the case of an inhibited sample the test may be repeated with a dilution of the DNA preparation at e.g. 1:10 (9 volumes Negative Control + 1 volume DNA Extract or eluted DNA). The Negative Control is used 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® Software, please inquire.
## F. Related and Accessory Products

<table>
<thead>
<tr>
<th>Product</th>
<th>Article No</th>
<th>Reactions</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kylt® RNA / DNA Purification</td>
<td>31314 / 31315</td>
<td>250 / 50</td>
<td>Combined RNA and DNA purification from veterinary samples</td>
</tr>
<tr>
<td>Kylt® Host Cells</td>
<td>31106 / 31107</td>
<td>100 / 25</td>
<td>Kit to detect animal host cells; to verify sample taking process</td>
</tr>
<tr>
<td>BCD 2x qPCR-Mix</td>
<td>31129</td>
<td>100</td>
<td>2-fold concentrated Real-Time PCR mix</td>
</tr>
</tbody>
</table>

Production:
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Development, manufacturing and distribution of Kylt® In-Vitro Diagnostica is certified according to ISO 9001:2015.

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**Prepare a Master-Mix**

* please refer to chapter 3

1. **Dispense Reaction- / Master-Mix and add 4 µl NC, DNA preparation, PC**

2. **Seal cavities, spin down (recommended), and start cycler**

3. **Analysis**

- **Normalized fluorescence**
- **Positive sample**
- **Negative sample**
- **Threshold**
- **CT**
- **Exponential phase**

PROTOCOL AT A GLANCE

Real-Time PCR Setup

Kylt® Products supplied with **Reaction Mix**

- 2.1 Thaw, if applicable
- 2.2 Pulse-vortex and spin down

Kylt® Products supplied with **Detection Mix**

- Pulse-vortex and spin down

+ 10 µl 2x qPCR-Mix (e.g. BCD 2x qPCR-Mix)

+ 6 µl Detection-Mix

* please refer to chapter 3