Kylt® ILT

Detection-Reagents specific for Infectious Laryngotracheitis Virus Real-Time PCR

www.kylt.eu
A. General

- Kylt® ILT Detection-Reagents are for the specific detection of viral DNA of Infectious Laryngotracheitis Virus (ILT, GaHV-1). The Detection-Reagents are to be combined with a Real-Time PCR-Mix (qPCR-Mix). The Detection-Reagents are suitable for the analysis of samples from birds, such as tissue and organs (e.g. trachea, lung, mucosal lesions of larynx and palpebra), swab samples (e.g. tracheal or lung) and sampling material derived from cultural processes with the aforementioned samples (e.g. cell culture harvest, chorioallantoic membrane).

- Kylt® ILT Detection-Reagents comprise the Detection-Mix and Positive and Negative Controls for detection of viral DNA of ILT. To conduct an analysis for ILT an additional, at least 2x concentrated qPCR-Mix is required, please inquire for recommendations. The qualitative testing with Kylt® ILT Detection-Reagents is based on a duplex Real-Time PCR: In one reaction setting, the target genes for ILT 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 ILT 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 ILT Positive Control per run the ILT-specific status of a sample can be evaluated in the end. This way, results can be achieved within a few hours after sample receipt.

- This detection system was developed for use by trained laboratory personnel following standardized procedures. This direction for use must be followed strictly.
B. Reagents and Materials

- Kylt® ILT Detection-Reagents contains the following reagents:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Color code of lid</th>
<th>Quantity 25 / 100 reactions</th>
<th>Storage conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5x ILT Detection-Mix</td>
<td>yellow</td>
<td>1 x / 4 x lyophilisate (final 230 µl each)</td>
<td>-18 °C to - 20 °C</td>
</tr>
<tr>
<td>ILT Positive Control</td>
<td>red</td>
<td>2 x / 4 x lyophilisate (final 20 µl each)</td>
<td>-18 °C to - 20 °C</td>
</tr>
<tr>
<td>Negative Control</td>
<td>blue</td>
<td>2 x 1 ml</td>
<td>-18 °C to - 20 °C</td>
</tr>
</tbody>
</table>

- After receipt, the components are immediately stored at -18 °C to -20 °C. Avoid repeated freezing and thawing of the components and keep them thawed as short as possible. If needed, generate appropriate aliquots upon its first thawing. The components are to be used within the indicated shelf life (see label on the outer packing), if stored properly. The components of different batches may not be mixed.

- Before its first use, rehydrate the ILT Positive Control: add 20 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 to -20 °C.

- The 2.5x ILT Detection-Mix needs to be stored protected from light at -18 °C to -20 °C. Do not expose to direct (sun) light. Before first use, rehydrate the lyophilized 2.5x ILT Detection-Mix: add 230 µl of the Negative Control per vial of 2.5x ILT Detection-Mix, briefly incubate at room temperature and mix by pulse-vortexing. Generate aliquots of suitable volumes and store them at -18 °C to -20 °C.

- The ILT-analysis can be processed on all commercially available Real-Time PCR thermal cyclers that can detect the emitted fluorescence of the fluorescent dyes FAM and HEX (except glass-capillary based thermal cyclers). The following Real-Time PCR thermal cyclers e.g. have been verified for routine diagnostics with this detection system: Mastercycler® RealPlex2 (Eppendorf), Rotor-Gene® 3000, 6000 & Q (Corbett / QIAGEN), Chromo4™ & CFX96 Touch™ (Bio-RAD), Applied Biosystems® 7500 & 7500 Fast (Life Technologies), Mx3005P (Stratagene / Agilent Technologies), Eco™ (Illumina®), qTower rapidPCR & qTower 2.0 (Analytik Jena), LightCycler® 96 (Roche).

- 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 equipment is needed (not included):
  - DNA preparation kit / protocol (e.g. NucleoSpin® Food, Macherey-Nagel)
  - qPCR-Mix (e.g. BCD 2x qPCR-Mix, BCD Baltic Customized Diagnostics GmbH, Germany)
  - Table top microcentrifuge
  - Vortex
  - Micropipettes covering volumes from 1 µl to 1000 µl
  - Centrifuge for PCR tubes or plates
  - Real-Time PCR thermal cycler
C. Control Reactions

- The included Negative Control provides the ability to exclude possible contaminations of the reagents. The included ILT Positive Control 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 ILT Positive Control reactions are included in every Real-Time PCR run and used to verify the validity of such run.

- The Internal Control is included in the 2.5x ILT Detection-Mix. In case of the absence of Real-Time PCR-inhibitors, the Internal Control can be detected in the Internal Control channel (HEX). This channel is used to confirm true-negative test results by excluding the presence of factors in the DNA extract that are inhibitory to Real-Time PCR.

D. Protocol

- The overall protocol of the ILT analysis consists of the following main workflow:
  1. sample processing
  2. DNA preparation
  3. reaction-setup and amplification
  4. data analysis – validity and qualitative result

1. Sample Processing

- We recommend pooling at most five samples or samples of five individuals, respectively, per DNA preparation.

- Samples 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 material is washed out thoroughly by pulse-vortexing and the washed out supernatant is used for DNA preparation. Single small swabs may directly be immersed in lysis buffer.

- Tissue samples are homogenized thoroughly in sterile buffer (see above) and a suitable volume is administered to DNA preparation.

2. DNA preparation

- 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. Reaction-Setup and Amplification

- Before each use, briefly vortex and spin down the 2.5x ILT Detection-Mix.

- To determine the total number of reactions needed, count the number of samples and add two more for the Negative Control and the ILT Positive Control.
Prepare the reaction mix for each sample 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 2.5x ILT Detection-Mix.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (µl) per reaction</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>qPCR-Mix (e.g. BCD 2x qPCR-Mix)</td>
<td>10 µl</td>
<td>1x</td>
</tr>
<tr>
<td>2.5x ILT Detection-Mix</td>
<td>8 µl</td>
<td>1x</td>
</tr>
<tr>
<td><strong>Total Master-Mix</strong></td>
<td><strong>18 µl</strong></td>
<td><strong>–</strong></td>
</tr>
<tr>
<td>Template (Negative Control / sample DNA preparation / Positive Control)</td>
<td>2 µl</td>
<td></td>
</tr>
<tr>
<td><strong>Total Reaction</strong></td>
<td><strong>20 µl</strong></td>
<td></td>
</tr>
</tbody>
</table>

When calculating volumes for preparation the Master-Mix consider the respective concentration of the qPCR-Mix used. For more information about the qPCR-Mix please refer to the Direction For Use of the respective manufacturer.

Add 18 µl of the Master-Mix to each of the PCR tubes or plate wells (“cavities”).

Exposed the 2.5x ILT Detection-Mix as short as possible to (sun)light and return it back to -18 °C to -20 °C right after application. Avoid the formation of bubbles when pipetting the Master-Mix, samples and controls.

Add 2 µl of the **Negative Control** to the corresponding cavity and seal it individually, if possible.

Add 2 µl of the **DNA preparation** to the corresponding cavity and seal it individually, if possible.

To minimize risk of potential cross-contaminations, 2 µl of the **ILT 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 ILT 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 start of the Real-Time PCR run.

Place the cavities in the Real-Time PCR thermal cycler and run the test. Please refer to the Direction For Use of the manufacturer of the qPCR-Mix for more details about the respective thermal profile that has to be applied. For example, when using BCD 2x qPCR-Mix the following parameters are applied for the test run:

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Duration</th>
<th>42 cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activation Polymerase</td>
<td>95 °C</td>
<td>10 min</td>
<td></td>
</tr>
<tr>
<td>Denaturation</td>
<td>95 °C</td>
<td>15 sec</td>
<td></td>
</tr>
<tr>
<td>Annealing &amp; Extension</td>
<td>60 °C</td>
<td>1 min</td>
<td></td>
</tr>
<tr>
<td>Fluorescence Detection</td>
<td>channels FAM and HEX</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Please follow the specified instructions of your Real-Time PCR thermal cycler as recommended by the manufacturer.

Depending on the qPCR-Mix used and its respective thermal profile this Kylt® ILT Detection-Reagents may be combined with further Kylt® Real-Time PCRs, such as Kylt® MGS Triplex. If detection systems for different pathogens are combined, please make sure to detect all relevant channels.

4. Data Analysis – Validity and Qualitative Results

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 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 ILT-specific status of each sample is analyzed (FAM).
Test Evaluation

- The **Real-Time PCR run** is only **valid** if the HEX-curve of the Negative Control 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 $\leq 35$ and the HEX-Ct-value of the Negative Control has to be $> 10$ and $\leq 40$.

<table>
<thead>
<tr>
<th>HEX-curve is positive</th>
<th>positive</th>
<th>negative</th>
<th>negative</th>
<th>negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>FAM-curve is negative</td>
<td>negative</td>
<td>positive</td>
<td>positive</td>
<td>negative</td>
</tr>
</tbody>
</table>

- The sample is **ILT negative** if its HEX-curve is positive ($10 < \text{Ct} \leq 40$), but its FAM-curve is negative.

- A **sample** is **positive** if its FAM-curve is significant positive ($10 < \text{Ct} \leq 34$), independent of the HEX-curve. A sample with a weak positive FAM-curve ($34 < \text{Ct} \leq 40$) has to be evaluated as **questionable**.

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

- **Recommendation:** In case of an inhibited sample you may repeat the test using e.g. a 1:10 and a 1:40 dilution of the respective DNA extract. The Negative Control serves as the diluting reagent. Alternatively, the entire DNA preparation process is repeated.

**Production:**

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