For in vitro Veterinary Diagnostics only.

Kylt® Porcine Rotavirus C

Real-Time RT-PCR Detection Kit for porcine Rotavirus Type C

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for porcine Rotavirus Type C

100 /25 reactions

in vitro Diagnosticum for swine

A. General

- Kylt® Porcine Rotavirus C Real-Time RT-PCR Detection Kit is for detection of Rotavirus Type C in samples from swine, such as swabs (e.g. rectal, from organs), organs and tissues (e.g. enteric mucosa, lymph nodes) and material derived from cultural processes with the aforementioned samples.

- Kylt® Porcine Rotavirus C comprises all reagents and controls for detection of viral RNA of Porcine Rotavirus C. The qualitative testing of Kylt® Porcine Rotavirus C is based on a duplex Real-Time RT-PCR system: The target genes specific for porcine Rotavirus Type C as well as for the housekeeping gene β-Actin (endogenous amplification control) are reverse transcribed (Reverse Transcription (RT)) and 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 RT-PCR). The probes specific for detection of target genes of amplified Rotavirus Type C and β-Actin 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 Rotavirus Type C 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® Porcine Rotavirus C 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>2x RT-qPCR-Mix</td>
<td>transparent</td>
<td>1 x / 4 x 280 µl</td>
<td>-18°C to -20°C</td>
</tr>
<tr>
<td>Detection-Mix</td>
<td>transparent</td>
<td>1 x / 4 x lyophilizate (final 150 µl each)</td>
<td>-18°C to -20°C</td>
</tr>
<tr>
<td>Positive Control</td>
<td>red</td>
<td>2 x / 4 x lyophilizate (final 50 µl each)</td>
<td>-18 °C to -20°C</td>
</tr>
<tr>
<td>Negative Control</td>
<td>blue</td>
<td>1 x 1 ml</td>
<td>-18 °C to -20°C</td>
</tr>
</tbody>
</table>

- After receipt the kit and its components are immediately stored at -18 °C to -20 °C. Avoid repeated freezing and thawing of the kit or its components and keep them thawed as short as possible. If needed, generate appropriate aliquots upon its first thawing. The kit and its 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.

- The Detection-Mix needs to be stored protected from light at -18 °C to -20 °C. Do not expose it to direct (sun-) light. Before first use the lyophilized Detection-Mix is rehydrated: add 150 µl of Negative Control per vial of Detection-Mix, briefly incubate at room temperature and mix by repeated vortexing. Generate aliquots of suitable volumes and store them at -18 °C to -20 °C.

- The reagents are suitable for the preparation and usage of a larger working Master-Mix. See recommendation in chapter 3 “Reaction Setup, Reverse Transcription and Amplification”.

- Before its first application rehydrate the lyophilized Positive Control by adding 50 µl of Negative Control per vial, briefly incubate at room temperature and mix by repeated vortexing. Generate aliquots of suitable volumes and store them at -18 °C to -20 °C.

- This kit can be used on all commercially available Real-Time PCR thermal cyclers that detect the emitted fluorescence of the fluorescent dye 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):
  - RNA extraction 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 RT and 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 RT-PCR run and used to verify the validity of such run.

- Kylt® Porcine Rotavirus C contains a primer/probe setup for the detection of β-Actin as Internal Control. In case of a successful RNA Isolation, no PCR inhibition and a correct sampling β-Actin will be detected in the HEX channel. The HEX channel therefore verifying the successful RNA Isolation as well as the absence of RNA inhibitors within the RNA preparation.

- It is recommended to run one or more of a RNA Isolation Control (RIC) per set of RNA preparations, depending on the total number of samples processes at once. The RIC is a »mock sample« composed of the plain sterile buffer used for raw sample processing. It is randomly placed between the samples, processed like a normal sample and enables for detection of potential contaminations of the reagents used for the RNA preparation (additionally to the Negative Control reaction, which monitors contaminations during PCR setup) as well as for detection of potential carryover contaminations between individual samples during the RNA preparation process.

D. Protocol

- The overall protocol of the analysis consists of the following main workflow:
  1. sample preparation
  2. RNA preparation
  3. reaction setup, Reverse Transcription 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 RNA preparation at -18 °C to -20 °C or -70 °C to -80 °C until further processing of the Rotavirus Type C detection. Avoid repeated freezing and thawing of the RNA preparations.

1. Sample Preparation

- We recommend pooling of not more than five individual samples or samples of more than five individuals, respectively, per RNA 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 and the washed out supernatant is used for RNA 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 RNA preparation.

2. RNA Preparation

- For detailed information on the RNA preparation process please refer to the manual of the respective kit.
3. Reaction Setup and Amplification

- Before each use, briefly vortex and spin down the 2x RT-qPCR-Mix and the rehydrated Detection-Mix (see also chapter B “Reagents and Materials”).

- To determine the total number of reactions needed, count the number of samples (including the RIC(s), if processed) and add two more for the Negative Control and the Positive Control.

- Prepare a Master-Mix containing the 2x RT-qPCR-Mix and the Detection-Mix for the appropriate number of reactions. Then add 16 µl of the Master-Mix to each of the PCR plate wells / cavities. The Real-Time RT-PCR is set up in the given order:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Per reaction</th>
<th>Volume (µl)</th>
<th>e.g. n=7</th>
</tr>
</thead>
<tbody>
<tr>
<td>2x RT-qPCR-Mix</td>
<td>10.0</td>
<td>70.0</td>
<td></td>
</tr>
<tr>
<td>Detection-Mix</td>
<td>6.0</td>
<td>42.0</td>
<td></td>
</tr>
<tr>
<td><strong>Total Master-Mix</strong></td>
<td><strong>16.0</strong></td>
<td><strong>112 µl, dispense 16 µl per reaction</strong></td>
<td></td>
</tr>
<tr>
<td>RNA (Negative Control / sample / Positive Control)</td>
<td>4.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total Reaction</strong></td>
<td><strong>20.0</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Recommendation**: Alternatively, a larger volume of a ready to use working Master-Mix can be prepared. The Master-Mix can be stored at -18 °C to -20 °C for at least six months and should be aliquoted in such a way that freeze-thaw-cycles are reduced to less than three.

- Expose the 2x RT-qPCR-Mix and the Detection-Mix as short as possible to (sun-)light and return both back to -18 °C to -20 °C 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 the sample RNA (final RNA preparations) to the corresponding cavity and seal it 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 spin them down before start of the Real-Time RT-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>Reverse Transcription</td>
<td>50 °C</td>
<td>10 min</td>
</tr>
<tr>
<td>Activation of Polymerase</td>
<td>95 °C</td>
<td>1 min</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95 °C</td>
<td>10 sec</td>
</tr>
<tr>
<td>Annealing &amp; Extension</td>
<td>60 °C</td>
<td>1 min</td>
</tr>
<tr>
<td>Fluorescence Detection</td>
<td>channel FAM and HEX</td>
<td>42 cycles</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® Porcine Rotavirus C Real-Time RT-PCR with other Kylt® Real-Time RT-PCRs. In the event of a combined Real-Time PCR run, 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 HEX-curve, respectively, in the linear increase of their slope (log scaling of the y-axis). 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 RT-PCR run. Afterwards, by means of the Internal Control ß-Actin 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 Rotavirus C-specific status of each sample is analyzed (channels FAM).

Test Evaluation

- The Real-Time RT-PCR test run is only valid if the FAM-curve of the Negative Control is negative and if the FAM-curve and HEX-curve of the Positive Control are 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>

<table>
<thead>
<tr>
<th>The sample is Rotavirus C</th>
<th>negative</th>
<th>positive</th>
<th>inhibited</th>
</tr>
</thead>
</table>

- A sample is negative for porcine Rotavirus C if its FAM-curve is negative (Ct > 42) and the HEX-curve is positive (Ct < 35).

- A sample is positive for porcine Rotavirus C if its FAM-curve is positive, independent of the HEX-curve.

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

- 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.