The Japanese encephalitis virus real-time RT-PCR kit is used for the detection of Japanese encephalitis virus in serum, plasma, C.S.F. or mosquito sample by using real time PCR systems.

2. Principle of Real-Time PCR
The principle of the real-time detection is based on the fluorogenic 5' nucleic acid. During the PCR reaction, the DNA polymerase cleaves the probe at the 5' end and separates the reporter dye from the quencher dye only when the probe hybridizes to the target DNA. This cleavage results in the fluorescent signal generated by the cleaved reporter dye, which is monitored real-time by the PCR detection system. The PCR cycle at which an increase in the fluorescence signal is detected is directly proportional to the amount of the specific PCR product. Monitoring the fluorescence intensities in real-time allows the detection of the accumulating product without having to re-open the reaction tube after the amplification.

3. Product Description
Japanese encephalitis is a mosquito-borne disease caused by the Japanese encephalitis virus. Over 50,000 cases are reported to occur each year. The disease is found in over 25 countries throughout the world. It occurs mainly in the rural and agricultural areas of Asia and Western Pacific. In temperate regions such as China, Japan and Korea, the transmission is highest from April to September. The disease is transmitted through the bite of an infected female culex mosquito. In the majority of cases these may occur within rural areas of the endemic countries and especially in regions where pig farming is found. The incubation period is usually five to 15 days. The majority of Japanese B encephalitis infections are subclinical (have no symptoms) and many infected people become asymptomatic carriers. Mild infections occur with few apparent symptoms other than fever with headache. Acute encephalitis can progress to paralysis, seizures, coma and death.

The Japanese encephalitis virus real-time RT-PCR kit contains a specific ready-to-use system for the detection of the Japanese encephalitis virus using RT-PCR (reverse transcription polymerase chain reaction) technology. The reaction is done in one step real-time RT-PCR. The first step is a reverse transcription (RT), during which the Japanese encephalitis virus RNA is transcribed into cDNA. Afterwards, a thermostable DNA polymerase is used to amplify the specific gene fragments by means of PCR (polymerase chain reaction). Fluorescence is emitted and measured by the real-time systems’ optical unit during the PCR. The detection of amplified Japanese encephalitis virus DNA is performed in fluorimeter channel FAM with the fluorochrome quencher BHQ1. In addition, the kit contains a system to identify possible PCR inhibition by measuring the HEV/XJC/OE fluorescence of the internal control (IC). An external positive control defined as 1x10³ copies/ml is supplied which allow the determination of the gene load. For further information, please refer to section 9.3 Quantification.

4. Kit Contents

<table>
<thead>
<tr>
<th>Ref</th>
<th>Type of reagent</th>
<th>Presentation</th>
<th>25 runs</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>JEV Super Mix</td>
<td>1 vial, 480µl</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>RT-PCR Enzyme Mix</td>
<td>1 vial, 28µl</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Molecular Grade Water</td>
<td>1 vial, 400µl</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Internal Control (IC)</td>
<td>1 vial, 30µl</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>JEV Positive Control (1x10³ copies/ml)</td>
<td>1 vial, 30µl</td>
<td></td>
</tr>
</tbody>
</table>

Analysis sensitivity: 5x10³ copies/ml
LOD: 1x10² - 1x10³ copies/ml

Note: Analysis sensitivity depends on the sample volume, elution volume, nucleic acid extraction methods and other factors. If you use the RNA extraction kits recommended, the analysis sensitivity is the same as it declares. However, when the sample volume is dozens or even hundreds of times greater than elution volume by some concentrating method, it can be much higher.

5. Storage
- All reagents should be stored at -20°C. Storage at +4°C is not recommended.
- All reagents can be used until the expiration date indicated on the kit label.
- Repeated thawing and freezing (> 3x) should be avoided, as this may reduce the sensitivity of the PCR reaction.
- Cool all reagents during the working steps.
- Super Mix should be stored in the dark.

6. Additionally Required Materials and Devices
- Biological cabinet
- Real time PCR system
- Desktop microcentrifuge for “eppendorf” type tubes (RCF max. 16,000 x g)
- Vortex mixer
- RNA extraction kit
- Real time PCR reaction tubes/plates
- Cryo-container
- Pipets (0.5 µl – 1000 µl)
- Sterile filter tips for micro pipets
- Sterile microtubes
- Disposable gloves, powderless
- Biohazard waste container
- Refrigerator and freezer
- Tube racks

7. ! Warnings and Precautions
- Carefully read this instruction before starting the procedure.
- For in vitro diagnostic use only.
- This assay needs to be carried out by skilled personnel.
- Clinical samples should be regarded as potentially infectious materials and should be prepared in a laminar flow hood.
- This assay needs to be run according to Good Laboratory Practice.
- Do not use the kit after its expiration date.
- Avoid repeated thawing and freezing of the reagents, this may reduce the sensitivity of the test.
- Once the reagents have been thawed, vortex and centrifuge briefly the tubes before use.

- Prepare quickly the Reaction mix on ice or in the cooling block.
- Set up two separate working areas: 1) Isolation of the RNA/ DNA and 2) Amplification/ detection of amplification products.
- Pipets, vials and other working materials should not circulate among working units.
- Use always sterile pipette tips with filters.
- Wear separate coats and gloves in each area.
- Do not pipette by mouth. Do not eat, drink, smoke in laboratory.

8. Sample Collection, Storage and transport
- Collected samples in sterile tubes.
- Specimens can be extracted immediately or frozen at -20°C to -80°C.
- Transportation of clinical specimens must comply with local regulations for the transport of etiologic agents.

9. Procedure
9.1 RNA-Extraction
RNA extraction kits are available from various manufacturers. You may use your own extraction systems or the commercial kit based on the yield. For the RNA extraction, please comply with the manufacturer’s instructions. The recommended extraction kit is as follows:

<table>
<thead>
<tr>
<th>Nucleic Acid Isolation Kit</th>
<th>Cat. Number</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA Isolation Kit</td>
<td>ML-0100MS/002</td>
<td>Qiagen</td>
</tr>
</tbody>
</table>

9.2 Internal Control
It is necessary to add internal control (IC) in the reaction mix. Internal Control (IC) allows the user to determine and control the possibility of PCR inhibition.

9.3 Quantitation
The kit can be used for quantitative or qualitative real-time RT-PCR.

For performance of quantitative real-time PCR, standard dilution must be prepared first as follows. Molecular Grade Water is used for dilution.

Dilution is not needed for performance of qualitative real-time PCR.

To generate a standard curve on the real-time system, all four dilution standards should be prepared as standards with specification of the corresponding concentrations.

Attention:
- Mix thoroughly before next transfer.
- The positive control (1x10³ copies/ml) contains high concentration of the target DNA. Therefore, be careful during the dilution in order to avoid contamination.

9.4 RT-PCR Protocol
The Master Mix volume for each reaction should be pipetted as follows:

- Prepare the Master Mix on ice (15µl volume).
- For use with ABI Prism 7000/7300/7500/7900/Step One Plus; IcyCycle (4ºC) (480ºM, Standard Cycling I: Bio-Rad CFX 96; RotorGene® 6000; MX3000P/2000P/MJ-Opticon2/Chromom4; LightCycler®480 instrument

Note: For ABI Prism 7000/7300/7500/7900/Step One Plus;

1. Run the negative control (IC) in the reaction mix. Internal Control (IC) allows the user to determine and control the possibility of PCR inhibition.
2. Add the internal control (IC) 1µl/µl and the result will be shown in the HEV/XJC/OE.
3. Quantitation
The Master Mix volume for each reaction should be pipetted as follows:

<table>
<thead>
<tr>
<th>Control</th>
<th>FAM</th>
<th>HEX/VIC/OE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular Grade Water</td>
<td>UNDET</td>
<td>25–35</td>
</tr>
<tr>
<td>Positive Control (quantitative assay)</td>
<td>≤35</td>
<td></td>
</tr>
<tr>
<td>QS (quantitative assay)</td>
<td>Correlation coefficient of QS curves: ≤0.98</td>
<td></td>
</tr>
</tbody>
</table>

1. PCR system without HEX/VIC/OE; channel may be treated with 1µl Molecular Grade Water instead of 1µl IC.
2. The volumes of Super Mix and Enzyme Mix per reaction multiply with the number of samples, which includes the number of controls, standards, and sample prepared. Molecular Grade Water is used as the negative control. For reasons of unspecific pipetting, always add an extra virtual sample. PCR Mix completely then spin down briefly in a centrifuge.
3. Pipet 20µl Master Mix with micropipets of sterile filter tips to each of the real-time PCR reaction plate/tubes. Separately add 5µl RNA sample template, positive and negative controls to different plate/tubes. Immediately close the plate/tubes to avoid contamination.
4. Spin down briefly in order to collect the Master Mix in the bottom of the reaction tubes.
5. Perform the following protocol in the instrument:

<table>
<thead>
<tr>
<th>Channel</th>
<th>Value</th>
<th>Selection of fluorescence channels</th>
</tr>
</thead>
<tbody>
<tr>
<td>FAM</td>
<td>1 cycle</td>
<td>HEX/VIC/OE</td>
</tr>
<tr>
<td>VIC</td>
<td>1 cycle</td>
<td>Target Nucleic Acid</td>
</tr>
<tr>
<td>VIC</td>
<td>1 cycle</td>
<td>HEX/VIC/OE</td>
</tr>
<tr>
<td>VIC</td>
<td>1 cycle</td>
<td>40 cycles</td>
</tr>
</tbody>
</table>

6. If you use ABI Prism® system, please choose “none” as passive reference and quencher.

10. Threshold setting: just above the maximum level of molecular grade water.

11. Calibration for quantitative detection: Input each concentration of standard controls at the end of run, and a standard curve will be automatically formed.

12. Quality control: Negative control, positive control, internal control and QS curve must be performed correctly, otherwise the sample results are invalid.

13. Data Analysis and Interpretation
The following results are possible:

<table>
<thead>
<tr>
<th>Value</th>
<th>Result Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>FAM</td>
<td>HEX/VIC/OE</td>
</tr>
<tr>
<td>≤35</td>
<td>Below the detection limit or negative</td>
</tr>
<tr>
<td>38</td>
<td>Positive; and the software displays the quantitative value</td>
</tr>
<tr>
<td>25–35</td>
<td>Re-test; if it is still 38–40, report as 38</td>
</tr>
<tr>
<td>UNDET</td>
<td>UNDET</td>
</tr>
</tbody>
</table>

Pipet 20μl Master Mix with micropipets of sterile filter tips to each of the real-time PCR reaction plate/tubes. Separately add 5μl RNA sample template, positive and negative controls to different plate/tubes. Immediately close the plate/tubes to avoid contamination.

For further questions or problems, please contact our technical support at trade@liferiver.com.cn.