1. Intended Use
TREPONEMA PALLIDUM (TP) Real-time PCR kit is used for the detection of TREPONEMA PALLIDUM (TP) in genital swabs or urine samples 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 assay. 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 inversely proportional to the amount of the specific PCR product. Monitoring the fluorescence intensities during Real Time allows the detection of the accumulating product without having to re-open the reaction tube after the amplification.

3. Product Description
TREPONEMA pallidum is a gram-negative spirochaete bacterium. It is the causative agent of syphilis. TREPONEMA pallidum is a spiral-shaped bacterium with outer and cytoplasmic membranes, a thin peptidoglycan layer, and periplasmic flagella. Mechanisms of T. pallidum pathogenesis are poorly understood. TREPONEMA pallidum is a helical to sinusoidal bacterium with outer and cytoplasmic membranes, a thin peptidoglycan layer, and periplasmic flagella. Mechanisms of T. pallidum pathogenesis are poorly understood.

4. Incubate the tube for 10 minutes at 100°C.
5. Prepare quickly the Reaction mix on ice or in the cooling block.
6. Avoid repeated thawing and freezing of the reagents, which may reduce the sensitivity of the test.
7. Pipets, vials and other working materials should be pipetted as follows:
A. Mix thoroughly before next transfer.
B. The positive control (1×10 copies/ml) contains high concentration of the target DNA. Therefore, be careful during the dilution in order to avoid contamination.
C. PCR Protocol
The Master Mix volume for each reaction should be pipetted as follows:

For negative controls, use 1µl of molecular grade water instead of 1µl of template DNA.

For positive controls, use 1µl of DNA extracted from laboratory strain T. pallidum, containing 1×10 copies/ml.

For each reaction:

1. Add 1 µl of template DNA to each reaction plate.
2. Pipet 38µl Master Mix containing 1µl of template DNA to each reaction plate.
3. Place the reaction plate in the PCR system.
4. Perform the following protocol in the instrument:

5. Analysis sensitivity: 1×10 copies/ml; LOQ: 2×10 copies/ml

Note: Analysis sensitivity depends on the sample volume, elution volume, nucleic acid extraction methods and other factors. If you use the DNA extraction buffer in the kit, 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.

2. Storage
All reagents should be stored at −20°C. Storage at +4°C is not recommended.

3. All reagents can be used until the expiration date indicated on the kit label.

4. Repeated thawing and freezing (>3 x) should be avoided, as this may reduce the sensitivity of the assay.

5. Cool all reagents during the working steps.

6. Reaction mix should be stored in the dark.

7. Additional Required Materials and Devices
- Biological cabinet
- Vortex mixer
- Pipetman
- Filter paper
- Sterile filter tips
- Sterile glass test tubes
- Refrigerator and Freezer
- Desktop microcentrifuge for "eppendorf" type tubes (RCF max. 16,000 x g)

8. 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 airflow hood.
This assay needs to be carried out by trained Laboratory Practice.
Do not use the kit after its expiration date.
Avoid repeated thawing and freezing of the reagents, which may reduce the sensitivity of the test.
Once the reagents are thawed, the samples and the PCR tubes should be used immediately.
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.
Avoid aerosols.

9. Procedure

9.1 DNA-Extraction
DNA extraction buffer is supplied in the kit, please thaw the buffer thoroughly and spin down briefly in the centrifuge before use. You may use your own extraction systems or commercial kits.

1) Wash the genital swabs in 1.0 ml normal saline and vortex vigorously. Centrifuge at 13000g for 5 minutes. Carefully remove and discard supernatant from the tube without disturbing the pellet.
2) Add 1.0ml normal saline and suspend the pellet with vortex vigorously. Centrifuge at 13000g for 5 minutes. Carefully remove and discard supernatant from the tube without disturbing the pellet.
3) Add 50µl DNA extraction buffer, close the tube then vortex for 10 seconds. Spin down briefly in a table centrifuge.
4) Incubate the tube for 10 minutes at 100°C.
5) Centrifuge the tube at 13000rpm for 5 minutes. The supernatant contains the DNA extracted and can be used for PCR template.

9.1.2 Urine sample
1) Take 1.5 ml of sample to a tube, Centrifuge the tube at 13000rpm for 2 minutes, carefully remove and discard supernatant from the tube without disturbing the pellet.
2) Add 1.0ml normal saline then vortex vigorously. Centrifuge the tube at 15000rpm for 5 minutes, carefully remove and discard supernatant from the tube without disturbing the pellet.
3) Add 50µl DNA extraction buffer, close the tube then vortex for 10 seconds. Spin down briefly in a table centrifuge.
4) Incubate the tube for 10 minutes at 100°C.
5) Centrifuge the tube at 13000rpm for 5 minutes. The supernatant contains the DNA extracted and can be used for PCR template.

Attention:
- During the incubation, make sure the tube is not open, for the vapor will volatilize into the air and may cause contamination in case the sample is positive.
- The extraction sample should be used in 3 hours or stored at -20°C for one month.
- DNA extraction kits are available from various manufacturers. You may use your own extraction systems or the commercial kit based on the yield. For DNA extraction, please comply with manufacturer's instructions.

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

The step of dilution is not needed for performance of qualitative real-time PCR.

Take positive control (1×10 copies/ml) as the starting high standard in the first tube. Respectively pipet 36µl Molecular Grade Water into next three tubes. Do three dilutions as the following figures:

<table>
<thead>
<tr>
<th>Dilution of Standards</th>
<th>Degree</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
</tr>
</tbody>
</table>

To generate a standard curve on the real-time system, all four dilution standards should be used and defined as standard with the corresponding concentrations.

Assuming:
A. Mix thoroughly before next transfer.
B. The positive control (1×10 copies/ml) contains high concentration of the target DNA. Therefore, be careful during the dilution in order to avoid contamination.

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

This system is only for Smart Cycler II

OR

PC system without HEX/VIC/OE channel may be treated with 1µl Molecular Grade Water instead of 1µl IC.

1. The volumes of the Enzyme 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 unprecise pipetting, always add an extra virtual sample. Mix completely then spin down briefly in a centrifuge.

2. Pipet 8µl Master Mix with micropipettes of sterile filter tips to each of the real-time PCR reaction plate/plates. Separately add 2µl DNA sample, positive and negative controls to different reaction plate/plates. Immediately close the plate/plates to avoid contamination.

3. Spin down briefly in order to collect the Master Mix in the bottom of the reaction tubes.

4. Perform the following protocol in the instrument:

1µl to 50µl reaction volume, selection of fluorescence channels

<table>
<thead>
<tr>
<th>Channel</th>
<th>C3 value</th>
<th>Result Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>FAM</td>
<td>HEX/VIC/OE</td>
<td>below detection limit or negative</td>
</tr>
<tr>
<td>VIC</td>
<td>HEX/VIC/OE</td>
<td>≥ 20</td>
</tr>
<tr>
<td>40 cycles</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1. If you use ABI Prism® system, please choose "melt" 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 standards at the end of each curve, 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 is invalid.

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

<table>
<thead>
<tr>
<th>C3 value</th>
<th>Result Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1P</td>
<td>UNDET</td>
</tr>
<tr>
<td>2P ≤ 20</td>
<td>positive, and the software displays the quantitative value</td>
</tr>
<tr>
<td>20 ≤ 30</td>
<td>re-test, if it is still 30~40, repeat as 1P</td>
</tr>
<tr>
<td>≥ 40</td>
<td>UNDET</td>
</tr>
</tbody>
</table>

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