HBV Quantitative & YMDD Mutation
Real Time PCR Kit
Cat. No.: HD-0003-01

For Use with LightCycler 1.0/LightCycler2.0/LightCycler480 (Roche)
Real Time PCR Systems

For In Vitro Diagnostic Use Only
User Manual

Shanghai ZJ Bio-Tech Co., Ltd.
www.liferiver.com.cn   Tel: +86-21-51320182
trade@liferiver.com.cn   Fax: +86-21-51320183
No.720 Cailun Road Zhangjiang High Technology Park, Shanghai, China
1. Intended Use
HBV quantitative and YMDD mutation real time PCR kit is used for the detection of HBV YMDD mutation in serum or plasma by using the real time PCR systems.

2. Introduction
Hepatitis B virus (HBV) is one of the most common infectious diseases in the world. More than 300 million people worldwide are estimated to have chronic HBV infection. Ten percent of these patients will die as a direct consequence of persistent viral infection. Nucleoside analogue therapy allows safe, long-term suppression of HBV and is a major milestone in the treatment of chronic hepatitis B. Lamivudine, the first of these agents approved worldwide, effectively suppresses viral replication, reduces disease activity, improves liver histology, and delays clinical progression. However, the development of lamivudine resistant mutations occurs in 14%-32% of patients after 1 year of therapy. The longer the treatment is continued, the more frequently resistance is seen (65% at 5 years). Therefore, it is necessary to monitor chronic hepatitis B patients before and during lamivudine treatment.

3. Principle of Real-Time PCR
The principle of the real-time detection is based on the fluorogenic 5’nuclease 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 initially (Ct) is 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.

4. Product Description
HBV quantitative and YMDD mutation real time PCR kit contains a specific ready-to-use system for the detection of HBV and HBV YMDD in one reaction using PCR (polymerase chain reaction) in the real-time PCR system. The master contains reagents and enzymes for the specific amplification of HBV DNA and HBV YMDD DNA fragment. Fluorescence is emitted and measured by the real time systems´ optical unit during PCR. The detection of amplified HBV DNA fragment for YMDD mutation is performed in fluorimeter channel FAM with the fluorescent quencher BHQ1. DNA extraction buffer is available in the kit and blood serum samples are used for DNA extraction. In addition, an external HBV-YMDD positive control (1 × 10^7 IU/ml) is contained in the kit to allow the determination of the gene load. For further information, please refer to section 10.2 Quantitation.

5. Kit Contents

<table>
<thead>
<tr>
<th>Ref.</th>
<th>Type of reagent</th>
<th>Presentation 25rxns</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DNA Extraction Buffer</td>
<td>1 vial, 1.8ml</td>
</tr>
<tr>
<td>2</td>
<td>HBV-YMDD Reaction Mix A</td>
<td>1 vial, 450µl</td>
</tr>
<tr>
<td>3</td>
<td>HBV-YMDD Reaction Mix B</td>
<td>1 vial, 450µl</td>
</tr>
<tr>
<td>4</td>
<td>PCR Enzyme Mix</td>
<td>1 vial, 22µl</td>
</tr>
<tr>
<td>5</td>
<td>Molecular Grade Water</td>
<td>1 vial, 400µl</td>
</tr>
<tr>
<td>6</td>
<td>HBV-YMDD Positive Control (1×10^7 IU/ml)</td>
<td>1 vial, 60µl</td>
</tr>
<tr>
<td>7</td>
<td>HBV-Y(I/V)DD Positive Control</td>
<td>1 vial, 60µl</td>
</tr>
</tbody>
</table>

6. 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 assay.
• Cool all reagents during the working steps.
• Reaction Mix should be stored in the dark.

7. 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
• Real time PCR reaction tubes/plates
• Cryo-container
• Pipets (0.5µl – 1000µl)
• Sterile filter tips for micro pipets
• Sterile microtubes
• Disposable gloves, powderless
• Biohazard waste container
• Refrigerator and Freezer
• Tube racks

8. Warnings and Precaution
Carefully read the instructions 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, and smoke in laboratory.
• Avoid aerosols

9. Sample Collection, Storage and transport
• Collect 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

10. Procedure
10.1 DNA-Extraction
DNA extraction buffer is supplied in the kit. Please thaw the buffer thoroughly and spin down briefly in the centrifuge before use.

1) Pipet 50µl blood serum to a 0.5ml tube, add 50µl DNA extraction buffer, closed the tube then vortex for 10 seconds. Spin down briefly in a table centrifuge.
2) Incubation the tube for 10 minutes at 100°C.
3) Centrifuge the tube at 13000rpm for 10 minutes. The supernatant contains the DNA extracted and can used for the PCR template.

**Attention:**
A. During the incubation, make sure the tube is not open, as the vapor will volatilize into the air and may cause contamination if the sample is positive.
B. The extraction sample should be used in 3 hours or store at -20°C for one month.
C. Different brand DNA extraction kits are available. You can also choose your own extraction systems or the commercial kit based on the yield. For the DNA extraction, please comply with the manufacturer’s instructions.

10.2 Quantitation
The kit can be used for quantitative or qualitative real-time PCR. A HBV-YMDD positive control defined as $1 \times 10^7$ IU/ml is contained in the kit.

For performance of quantitative real-time PCR, standard dilutions must be prepared first as follows. Molecular Grade Water is used for dilution.
**Dilution is not needed for performance of qualitative real-time PCR.**

Take positive control ($1 \times 10^7$ IU/ml) as the starting high standard in the first tube. Respectively pipette 36ul of Molecular Grade Water into next three tubes. Then do three dilutions as the following figures:

![Dilution of Standards](image)

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

**Attention:**
A. Mix thoroughly before next transfer.
B. The positive control ($1 \times 10^7$ IU/ml) contains high concentration of the target DNA. Therefore, be careful during the dilution in order to avoid contamination.

10.3 PCR Protocol
The Master Mix volume for each reaction should be pipetted as follows:
1) The volumes of Reaction 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. *(n: the number of reaction).*

<table>
<thead>
<tr>
<th>Reaction Volume</th>
<th>Master Mix Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>18µl Reaction Mix</td>
<td>18µl × (n+1)</td>
</tr>
<tr>
<td>0.4µl Enzyme Mix</td>
<td>0.4µl × (n+1)</td>
</tr>
</tbody>
</table>

Mix completely then spin down briefly in a centrifuge.

2) Pipet **18µl** Master Mix A with micropipets of sterile filter tips to each of the *Real time* PCR reaction plate/tubes. Separately add **2µl** DNA sample, positive and negative controls to different reaction plate/tubes. Immediately close the plate/tubes to avoid contamination.

3) Pipet **18µl** Master Mix B with micropipets of sterile filter tips to each of the *Real time* PCR reaction plate/tubes. Separately add **2µl** DNA sample, positive and negative controls to different reaction 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:

- **37°C** for **2 min, 1 cycle**;

- **94°C** for **2 min, 1 cycle**;
93°C for 15 sec, 60°C for 30 sec, 40 cycles. 

Fluorescence is measured at 60°C

11. Data Analysis and Interpretation
The following results are possible:
1) HBV-YMDD positive control is detected positive both in Reaction Mix A and Reaction Mix B of channel FAM, while HBV-Y(I/V)DD positive control is detected positive in Reaction Mix A and negative in Reaction Mix B of channel FAM.
2) Signals are detected both in Reaction Mix A and Reaction Mix B of channel FAM. The result is positive: The sample contains HBV DNA and is YMDD wild type.
3) A signal is detected in Reaction Mix A of channel FAM but no signal is detected in Reaction Mix B of channel FAM. The result is positive: The sample contains HBV DNA and it is YIDD or YVDD mutant type.
4) No Signals are detected both in Reaction Mix A and Reaction Mix B of channel FAM. The sample does not contain any HBV DNA. It can be considered negative.

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