

HBV mutant (YMDD) Test Kit (RFLP Technology)

Cat. No. C02-01-1114

PCR-RFLP detection of Hepatitis B virus wildtype and mutant strains

Includes main components for 150 reactions



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HAI KANG LIFE CORPORATION LIMITED

PLEASE READ THROUGH THE ENTIRE PROTOCOL BEFORE STARTING.

1. KIT COMPONENTS

The kit contains reagents for a total of 150 PCR reactions:

- 4 x 530 µl PCR mastermix 1 (with primer 1, store at -20°C)
- 4 x 530 µl PCR mastermix 2 (with primer 2, store at -20°C)
- 4 x 530 µl PCR mastermix 3 (with primer 3, store at -20°C)
- 1 x 50 µl Positive Control 1 (store at -20°C)
- 1 x 50 µl Positive Control 2 (store at -20°C)
- 1 x 50 µl Positive Control 3 (store at -20°C)

Additional reagents required (all molecular grade)

- DNA extraction kit for blood (QIAamp DNA Blood Kit (cat No. 51104) or other method giving DNA of similar PCR quality)
- Restriction enzymes Fok1, SspI, Alw441
- 2.55 M potassium or sodium acetate, pH 4.8
- Absolute ethanol

Storage Conditions

The mastermixes should be stored at -20°C.

Note: Please aliquot the mastermix solutions into appropriate volume according to your test frequency, in order to minimize repeated freeze and thaw cycles. Frequent thawing and freezing may inactivate some kit components.

2. PROCEDURES

A. DNA Extraction From Blood

Method

Centrifuge the blood sample at 800 x g for 10 min to spin down blood cells. Collect the upper clear layer (serum for clotted blood, or plasma for whole blood) for testing.

Precaution: store blood sample in 4°C refrigerator and never freeze clotted blood.

Follow instructions of Qiagen Blood Protocol with exception of final elution step: Add 100ml **AE** or distilled water to the QIAamp spin column, incubate for at least 5 min at room temperature. Centrifuge at 6000 g at 25°C for 1 min to collect DNA from the sample.

B. PCR

Note: Thaw kit reagents just before use. Mix thawed reagents thoroughly. Do not vortex the enzyme-containing mastermix. Only thaw as many PCR mastermix tubes as are required.

3 sets of PCR reactions using PCR mastermix 1, 2, and 3 are required for testing.

Each set includes patient sample(s), positive (control DNA) and negative controls.

- Prepare the Reaction Mix in a sterile 0.5 ml microfuge tube as follows:

Components	Volume per reaction
dd Water	31.8 µl
PCR mastermix 1, 2 or 3	13.2 µl
Total Volume	45 µl

- Aliquot 45 µl reaction mix to each tube.
- Add 5 µl sample DNA (if using Qiagen Blood DNA extraction kit) to each corresponding tube.

At least 9 PCR reactions are required for each patient sample with the 3 sets of PCR mastermixes, including patient sample and control DNA:

	Number of Reactions		
	PCR mastermix 1	PCR mastermix 2	PCR mastermix 3
Patient Sample	1	1	1
Positive Control	1	1	1
Negative Control (dd Water)	1	1	1
Total	3	3	3

- When all PCR reactions are set up, load all PCR tubes into the PCR thermal cycler and use the cycling conditions shown below:

1 Cycle	94°C	5 Minutes
40 Cycles	94°C	30 Seconds
	52°C	45 Seconds
	72°C	30 Seconds
1 Cycle	72°C	10 Minutes

C. Gel Electrophoresis (Part 1)

- Prepare a 2% agarose gel in 1X Tris-Acetate-EDTA (TAE) buffer.
- Load PCR products (10 µl + loading dye) into each well and perform gel electrophoresis at 24 mA constant until dye front reaches about half of the gel length.
- PCR Product information:

PCR Amplicon size (bp)		
Primer 1	Primer 2	Primer 3
181	274	138

D. PCR products purification

If the duplicated patient samples give visible PCR amplicon on the gel, the PCR amplicon should be purified before proceeding to restriction enzyme digestion.

- Transfer PCR products (~40 µl remaining) to a 0.5ml microfuge tube.
- Add 1/10 volume (~4 µl) of 2.55M potassium acetate to the PCR products.

- Add 2.5 fold volume (~100 µl) of absolute ethanol to the mixture and mix well by vortex.
- Stand at room temperature for at least 15 min.
- Centrifuge at max speed for 10 min at room temperature. Discard the supernatant carefully and air dry for at least 10 min.
- Add 24 µl dd water to each tube to resuspend the pellet.

E. Restriction Enzyme Digestion

- After PCR product purification, prepare the components for restriction enzyme digestion:

Component	Enzyme		
	<i>Alw441</i> (<i>ApaL1</i>)	<i>FokI</i>	<i>SspI</i>
Purified PCR product	24 µl (Primer 1)	24 µl (Primer 2)	24 µl (Primer 3)
Buffer 10x*	3 µl	3 µl	3 µl
0.1% BSA#	3 µl	3 µl	3 µl
Final volume	30 µl	30 µl	30 µl

*Check the buffer requirement of the restriction enzymes purchased.
#If BSA is not required for the enzyme purchased, substitute the volume with dd water.

- Mix gently and then divide the mixture into two tubes:

Restriction enzyme	Primer 1		Primer 2		Primer 3	
	<i>Alw441</i> (<i>ApaL1</i>)	<i>FokI</i>	<i>FokI</i>	<i>SspI</i>	<i>SspI</i>	<i>SspI</i>
PCR product mixture from step 1	15	15	15	15	15	15
Restriction enzyme	5 U	- (uncut)	5 U	- (uncut)	5 U	- (uncut)

- Incubate the tubes in a waterbath at 37°C for 1 hour.

F. Gel Electrophoresis (Part 2)

- Prepare a 3% agarose gel in 1X Tris-Acetate-EDTA (TAE) buffer
- Immerse the gel in electrophoresis tank containing 1X TAE buffer.
- Load PCR products (15 µl + loading dye) into each well.

Perform gel electrophoresis at 24 mA constant current until dye front reaches about two-thirds of the gel length.

3. DATA ANALYSIS AND INTERPRETATION

By comparing the size of the bands with DNA size markers and with the YMDD controls, the presence of mutant genes in the patient sample can be inferred.

- Before restriction enzyme digestion:

PCR amplicons not digested by restriction enzyme produce three distinctly sized amplification products as shown in section 2C.

HBV Variant	Amplicon size (bp)		
	Primer 1	Primer 2	Primer 3
YMDD	181	274	138
YVDD	181	274	138
YIDD	181	274	138

- Restriction enzyme digestion products:

Restriction enzyme digestion of PCR amplicons from the three primer sets produce distinctly sized fragments for each HBV variant.

HBV Variant	Restriction digest products		
	Primer 1 <i>Alw441</i>	Primer 2 <i>FokI</i>	Primer 3 <i>SspI</i>
YMDD	181 bp	174 + 100 bp	138 bp
YVDD	158 + (23) bp	174 + 100 bp	138 bp
YIDD	181 bp	274 bp	109 + (29) bp

YMDD: The PCR product of the YMDD variant is not digested by *Alw441* or *SspI*. However, the 274 bp amplicon from Primer 2 is digested by *FokI* to produce fragments of 174 bp and 100 bp in size.

YVDD: The amplified product of the YVDD variant is not digested by *SspI*. However, the 181 bp amplicon from Primer 1 is digested by *Alw441* to produce the 158 bp and 23 bp fragments. The 274 bp amplicon from Primer 2 is digested by *FokI* to produce fragments of 174 bp and 100 bp in size.

YIDD: The amplified product of the YIDD variant is not digested by *Alw441* or *FokI*. However, the 138 bp amplicon from Primer 3 is digested by *SspI* to produce fragments of 109 bp and 29 bp in size.

Control DNA PCR products should give 158 + 23 bp fragments by *Alw441*, 174 + 100 bp fragments by *FokI*, and 109 + 29 bp fragments by *SspI*.

Note: The 23 bp and 29 bp fragments may not be visible on 3% gel.

4. TECHNICAL ASSISTANCE

Our technical staff will provide technical assistance you may need in using this kit. Simply call +(852) 2111 2123 during office hours:

Monday – Friday: 9:00am to 5:30pm
Saturday: 9:00am to 1:00pm

A recorded message (in English, Cantonese or Putonghua) may be left outside office hours.

Alternatively, you may contact our technical staff by fax or email.

Fax: +(852) 2111 9762
Email: technical@haikanglife.com

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