Instruction Manual **§ Research use only §**

IMMUNIS® HBV Genotype EIA

EIA kit for serological determination of hepatitis B virus genotype

- This kit is intended for research use only, not for diagnostic use.
- Read this instruction manual thoroughly before use. This kit must be used according to the instructions and for the purpose described in this manual. No result is guaranteed in any use or for any purpose other than those described in this manual.
- The positive control of this kit is HBsAg positive, which is potentially infectious, whereas tested negative to anti-HCV and anti-HIV1/2. Universal precautions are required to handle controls as well as test sera.

I. Background and assay principle

There are eight genotypes (genotype A to H) of hepatitis B virus (HBV) which are distinguished by > 8 % difference in the nucleotide sequence of the HBV genome [1, 2]. Genotypes A and D are common in Europe and the United States, while genotypes B and C are prevalent in Asia. Genotype F is frequent in Latin America, while genotype E is confined in Central and West Africa. Many studies indicate that HBV genotype influence the severity of chronic hepatitis B and response to interferon therapy. HBV genotypes have been determined by tedious methods such as direct sequencing, genotype-specific PCR, PCR-RFLP, line-probe assay (LiPA), or PCR-invader assay.

"IMMUNIS ® HBV Genotype EIA" is an enzyme immunoassay (EIA) based HBV genotyping kit, which designed to discriminate between genotypes to A, B, C, or D by detecting genotype-specific epitopes in PreS2 region [3-5].

HBs antigen (HBsAg) possesses some of four genotype dependent epitopes (called 'm', 'k', 's', and 'u') in its PreS2 region. Genotype A, B, C, or D virus present 'su', 'm', 'ks', or 'ksu' epitopes respectively. Therefore, genotypes can be determined serologically by the positive/negative combinations (serotypes) of four EIA that is specific for epitopes 'm', 'k', 's', or 'u', respectively.

This kit adopts one-step sandwich EIA for HBV genotyping. In the first step, HRP-labeled anti-'m', -'k', -'s', or -'u' mAb followed by sample sera are added to the anti-HBs coated wells. During 1st reaction, HBsAg in the sample form complex with HRP-labeled mAb and captured by anti-HBs coated wells. The wells are washed, and the color is developed with HRP substrate.

II. Kit components

1. Microplate coated with anti-HBs (8 wells x 12 strips) ..... 2 plates
   (Anti-HBs mouse monoclonal antibody)
2. HRP-labeled mAbs (HRP-labeled anti-PreS2 mouse monoclonal antibodies)
   1) HRP-labeled anti-'m' mAb (green) ..... 5 mL x 1 vial
   2) HRP-labeled anti-'k' mAb (yellow) ..... 5 mL x 1 vial
   3) HRP-labeled anti-'s' mAb (blue) ..... 5 mL x 1 vial
   4) HRP-labeled anti-'u' mAb (red) ..... 5 mL x 1 vial
3. Negative control ..... 1 mL x 1 vial
4. Positive controls
   1) Positive control 'm' (green) ..... 0.25 mL x 1 vial
   2) Positive control 'k' (yellow) ..... 0.25 mL x 1 vial
   3) Positive control 's' (blue) ..... 0.25 mL x 1 vial
   4) Positive control 'u' (red) ..... 0.25 mL x 1 vial
5. HRP substrate (3, 3', 5, 5'-tetramethylbenzidine) ..... 20 mL x 1 vial
6. Reaction stopper ..... 20 mL x 1 vial
7. Washing solution (20x concentrated) ..... 50 mL x 2 vials
8. Plate seal ..... 6 sheets

III. Application

Hepatitis B Virus serological genotyping in human serum
(Genotype A, B, C, or D)

IV. Operational precautions

1. Sample collection and storage

1) When collecting blood, avoid hemolysis and separate serum immediately.
2) It is recommended to use fresh sera for the assay. Keep frozen if they are stored and avoid frequent freeze-thaw cycles. Thaw the serum samples to room temperature and mix before use.

2. Interference substances

1) Up to 482 mg/dL of hemoglobin, 18.2 mg/dL of bilirubin F, 20.1 mg/dL of bilirubin C, 1,670 FTU of chyle, or 50 IU/mL of rheumatoid factor did not interfere with the determination.

3. Operational precautions

1) Use clean equipment for the assay.
2) Replace micropipette tips for each sample and reagent.
3) Assay with Negative control and Positive controls in each assay.
4) Once assay is started, complete it within the prescribed time, allowing the same reaction time for all samples. Do not dry up the inner surface of the wells during operation.
5) Measure absorbance within 30 min after stopping the color development.
6) Do not scrape or touch the bottom of wells.

V. Operation

1. Preparation of reagent

1) Washing solution
   Dilute Washing solution (20x concentrated) 20 times with purified water. Store diluted solution at 1 ~ 30ºC and use within 1 month.
2) Return reagents of the kit to 20 ~ 35ºC, and mix well before use.
3) The microplate can be divided into 12 strips. Strips not used should be kept in an aluminum pouch together with desiccant and stored at 2 ~ 10ºC.

2. Materials required but not provided

1) Micropipettes, 20 µL and 100 µL
2) A microplate shaker
3) A microplate washer
4) A microplate reader (main wavelength 440 ~ 460 nm, sub wavelength 550 ~ 750 nm)

3. Assay procedure

Note: Layout test wells as illustrated below. Assay with four wells for 4 epitopes per a sample. Use two wells for Negative control and 1 well for Positive control to each epitope.

An example of well layout

epitope	'm' 1	'k' 2	's' 3	'u' 4	'm' 5	'k' 6	's' 7	'u' 8
A	NC	NC	NC	NC	S6	S6	S6	S6
B	NC	NC	NC	NC	S7	S7	S7	S7
C	PC m	PC k	PC s	PC u	S8	S8	S8	S8
D	S1	S1	S1	S1	S9	S9	S9	S9
E	S2	S2	S2	S2	S10	S10	S10	S10
F	S3	S3	S3	S3	:	:	:	:
G	S4	S4	S4	S4	:	:	:	:
H	S5	S5	S5	S5	:	:	:	:

NC : Negative control
PC : Positive controls
S1 ~ S10... : Sample sera 1 ~ 10...

1) Addition of HRP-labeled mAbs
   Pipette 100 µL of HRP-labeled anti-'m', -'k', -'s', or –'u' mAb to each wells according to the well layout.
2) Addition of controls and samples
   According to the well layout, dispense 20 µL of Negative control to 8 wells, Positive control 'm', 'k', 's', and 'u' to 1 well each, and serum samples to each 4 wells (m, k, s, u) respectively.
3) 1st reaction
   Cover the microplate wells with the plate seal and shake it at 20 ~ 35ºC for 2 hours on a microplate shaker.
4) Washing
   Remove the plate seal and wash all wells 5 times with 350 µL of the washing solution prepared under **"1) Washing solution"** in **"1. Preparation of reagent"**. Then tap the microplate upside down on a clean paper towel.
   Follow the next step immediately so as to avoid drying up the wells.
5) Addition of HRP substrate
   Pipette 100 µL of HRP substrate to each well.
6) Enzyme reaction
   Cover the microplate with new plate seal and leave the microplate at 20 ~ 35ºC in the dark for 30 min.
7) Addition of Reaction stopper
   Remove the plate seal and stop color development by adding 100 µL of Reaction stopper in all wells, and mix sufficiently until the colors changed from blue to yellow.
8) Absorbance measurement
   Measure the absorbance of each well at 450 (440 ~ 460) nm (with reference at 550 ~ 750 nm in two-wavelength mode).
   Absorbance should be measured within 30 min after stopping the reaction.

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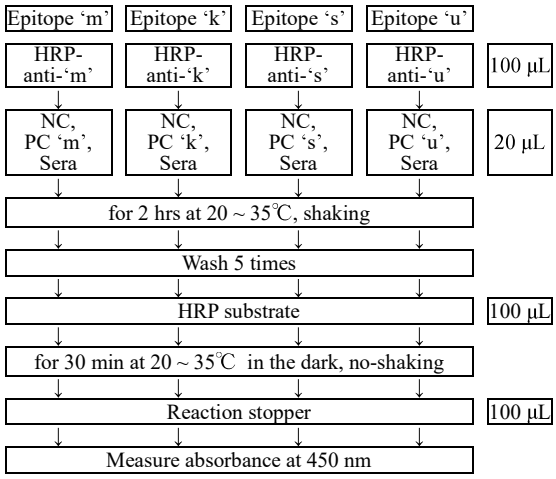
Scheme of assay procedure



VI. Determination of HBV genotypes from assay results

1. Determination of positive/negative result for each epitope

1) A_{450} of the negative controls should be < 0.1.
2) A_{450} of the positive control for each epitope should be > 0.5.
3) Calculate the cut-off value (COV) for each epitope
   COV = (mean A_{450} of the negative controls) + 0.05
4) Positive and negative results of each epitopes
   Negative (-); A_{450} values < COV
   Positive (+); A_{450} values ≥ COV

2. Re-test

1) When A_{450} value of one positive epitope is less than 1/10 of the maximum A_{450} value in other positive epitopes, serotype/genotype determination of the sample should be suspended, and then assay again with 10 times diluted test serum.

An example for re-test;

Epitope	m	k	s	u
A_{450}	0.040	**3.500**	**3.500**	**0.100**
COV	0.060	0.060	0.060	0.060

In this case, A_{450} of epitope 'u' is over its COV, but is under 1/10 of those of epitopes 'k' and 's'. Therefore, the serotyping should be suspended, NOT be 'ksu'.

2) Sample dilution; Dilute 10 µL of sample sera with 90 µL of washing solution.
3) Assay again according to **"V. Operation"**.
4) If any A_{450} value of the positive epitopes in the re-test correspond to **"1)"** in **"2. Re-test"** above, HBV genotype of the sample cannot be classified by this kit.

3. Determination of HBV genotypes

Determine genotypes by the positive/negative combinations (serotypes) of four epitopes ('m', 'k', 's', or 'u') in accordance with the table below. Sample sera showing serotypes other than 'su', 'm', 'ks', or 'ksu' can not be classified by this kit.

PreS2 epitope				PreS2 serotype	HBV genotype
m	k	s	u		
−	−	+	+	su	A
+	−	−	−	m	B
−	+	+	−	ks	C
−	+	+	+	ksu	D
				Others	Unclassified

4. Precaution in determination

1) Sensitivity;
Of the sera with HBsAg ≥ 3 IU/mL, 98 % were determined to genotypes A, B, C, or D, however, sera with lower HBsAg concentrations may not be detectable for any of the four epitopes.

2) Precision;
In 91 samples that have been genotyped by DNA sequencing, 87 (95.6 %) were in complete accordance with this EIA genotyping, and the other 4 (4.4 %) were 'Unclassified' [5].

		Genotyping by this EIA kit					Total	Accordance
		A	B	C	D	UC*		
Genotyping by sequencing	A	19				1	20	95.0 %
	B		20			1	21	95.2 %
	C			40		2	42	95.2 %
	D				8		8	100.0 %
Total		19	20	40	8	4	91	95.6 %

*UC; Unclassified

3) Genotypes E, F, G, H and sub-genotypes;
Results of sub-genotypes and genotypes E, F, G, and H are not elucidated well, while Tanaka reported that samples of sub-genotypes Aa, Ae, Ba, Bj, C1, and C2 are genotyped accurately, furthermore genotype E, and G results in serotype 'ksu', which is classified to genotype D, genotype H results in serotype 'ks' to genotype C by the EIA [5]. Usuda showed genotype F leads to serotype 'k' [3], which is 'Unclassified' in this kit.

Genotype/ Subgenotype	Results of the EIA	
	Serotype	Genotype
Aa	'su'	A
Ae	'su'	A
Ba	'm'	B
Bj	'm'	B
C1	'ks'	C
C2	'ks'	C
D	'ksu'	D
E	'ksu'	D
F	'k'	Unclassified
G	'ksu'	D
H	'ks'	C

4) Abnormal results;
Despite their high HBsAg titers, rare sera can show serotypes which do not correspond to any of genotypes A, B, C, or D. They may have mutations in the PreS2-region which result in the modification of PreS2 epitopes. Or else, they may contain HBsAg of two or more genotypes, due to double (or multiple) infection with HBV of different genotypes.

VII. Warnings and precautions

1. General precautions

1) Assay strictly as instructed.
2) Do not use expired reagents.
3) Do not mix up kit components of different production lots.
4) Avoid contamination of the kit reagents with microorganisms.
5) Close the lid of the reagent container tightly after use.

2. Handling precautions

1) Sera containing HBsAg are potentially infectious with HBV, HCV, and HIV. Take care not to touch samples with bare hands or let them splash into the eye. Use disposable gloves in handling samples, and wash your hands thoroughly after completing the test.
2) Avoid contact of reagents. If they contact skin, wash with plenty of water. Get medical care if necessary.
3) Test sera, reagents and the materials used for the assay should be treated with either of the followings.
   a) Immerse in 0.05 w/v% formalin solution at 37℃ for over 72 hrs.
   b) Immerse in 2 w/v% glutaraldehyde solution for over 1 hr.
   c) Immerse in sodium hypochlorite solution (concentration of effective chlorine: 1,000 ppm or more) for over 1 hr.
   d) Autoclave at 121℃ for 20 min.

VIII. Storage and shelf life

Store the kit at 2 ~ 10℃ and avoid freezing.
This kit is stable for 1 year after the date of manufacture. Validity of kit is shown in the package.

IX. Package

1 kit for 48 tests Code No. 1A65

X. Reference

1) Okamoto H, Tsuda F, Sakugawa H, et al: Typing hepatitis B virus by homology in nucleotide sequence: comparison of surface antigen subtypes. J Gen Virol **69**: 2575-2583, 1988.
2) Arauz-Ruiz P, Norder H, Robertson BH, et al: Genotype H: a new Amerindian genotype of hepatitis B virus revealed in Central America. J Gen Virol **83**: 2059-2073, 2002.
3) Usuda S, Okamoto H, Iwanari H, et al: Serological detection of hepatitis B virus genotypes by ELISA with monoclonal antibodies to type-specific epitopes in the preS2-region product. J Virol Methods **80**: 97-112, 1999.
4) Moriya T, Kuramoto IK, Yoshizawa H, et al: Distribution of hepatitis B virus genotypes among American blood donors determined with a PreS2 epitope enzyme-linked immunosorbent assay kit. J Clin Microbiol. **40**: 877-880, 2002.
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