

HBV Genotype EIA

(CODE: 1A64)

For Serological Determination of Hepatitis B Virus Genotypes

A kit contains microplates and color-coded reagents for 48 tests, along with positive and negative controls, and reagents for enzyme reaction and washing solution.



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BACKGROUND AND THE PRINCIPLE OF "HBV GENOTYPE EIA"

There are six genotypes of hepatitis B virus (HBV) which are distinguished by > 8% difference in the nucleotide sequence of the entire genome (~3200 nucleotides). They are called genotypes A, B, C, D, E and F.^{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 the New World, while genotype E is confined in Central and West Africa. Because HBV strains of genotype E cluster with those of genotype D in comparison of the X-gene sequence,³ the independence of genotype E has been questioned. There have been increasing lines of evidence to indicate that HBV genotypes may influence the severity of chronic hepatitis B and response to interferon therapy.^{4,5} Studies on HBV genotypes have been hampered, however, by tedious methods for their classification which depend on the nucleotide sequence, such as restriction fragment length polymorphism and polymerase chain reaction with genotype-specific primers.

Because the nucleotide sequence of preS2 region can vary widely, depending on various genotypes, epitopes expressed on the preS2-region product are instrumental for distinguishing genotypes.⁶ Five monoclonal antibodies (mAbs) were raised against the preS2-region products of HBV isolates of various genotypes. The epitope b is expressed commonly by hepatitis B surface antigen (HBsAg) of all genotypes. Expressions of the other four epitopes (m, k, s and u), recognized by mAbs, are characteristic of the five major HBV genotypes (A, B, C, D and F). Therefore, these five major genotypes can be determined serologically by the combination of preS2 epitopes detected on HBsAg by EIA.

"HBV Genotype EIA" involves three steps (see figure below). In the first step, HBsAg in the test serum is captured by wells of a microplate coated with mAb to the common determinant 'a' of HBsAg. The plate is washed. Then, in the second step, each of the four wells (for a test) receives enzyme-labeled mAb to epitope m, k, s or u (color coded). The plate is washed, and the color is developed in wells. In the third and last step, genotypes are determined by the combination of preS2 epitopes (serotypes) recognized by mAbs: "su" for genotype A, "m" for genotype B, "ks" for genotype C, "ksu" for genotype D, and "k" for genotype F.

Epitope b is expressed on the preS2-region products of all genotypes. Hence, it needs not to be determined in serological differentiation of genotypes. The detection of epitope b guarantees the presence of the preS2-region product which is prerequisite to the serological genotyping. This is sometimes necessary, because the preS2-region products are easily digested with contamination with bacteriae or proteases in the test serum.

MATERIALS PROVIDED

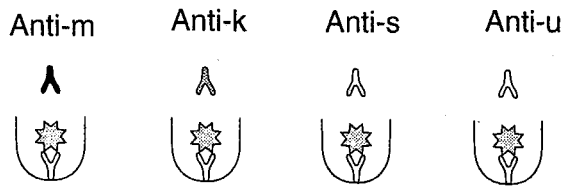
One negative and five positive controls, as well as five mAbs to PreS2 epitopes, are color coded.

1. Microplate (8 x 12 wells) coated with mAb to the common determinant 'a' of HBsAg x 2
2. Negative control for all epitopes (black) 1.5 mL x 2

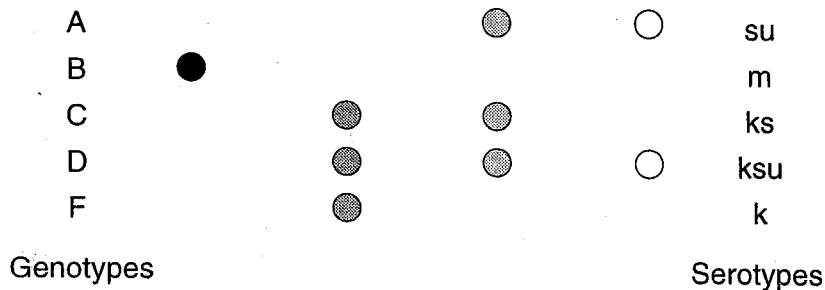
STEP 1: Capture of HBsAg (★) in Wells Coated with Anti-HBs (Y)



STEP 2: Binding of Enzyme-Labeled mAb to PreS2 Epitopes



STEP 3: Determination of the Five Major HBV Genotypes



3. Positive control for epitope b (white) 0.5 mL x 2
4. Positive control for epitope m (green) 0.5 mL x 2
5. Positive control for epitope k (yellow) 0.5 mL x 2
6. Positive control for epitope s (blue) 0.5 mL x 2
7. Positive control for epitope u (red) 0.5 mL x 2
8. Diluent (fetal calf serum) 15 mL x 1
9. Labeled mAb to epitope b (white) 3 mL x 1
10. Labeled mAb to epitope m (green) 3 mL x 1
11. Labeled mAb to epitope k (yellow) 3 mL x 1
12. Labeled mAb to epitope s (blue) 3 mL x 1
13. Labeled mAb to epitope u (red) 3 mL x 1
14. Substrate for color development 10 mL x 1
(H₂O₂ and tetramethylbenzidine)
15. Reaction stopper 10 mL x 1
16. Washing solution (20-times concentrated) 25 mL x 4
17. Plastic adhesive films for sealing microplate during reaction x 10

MATERIALS REQUIRED BUT NOT PROVIDED

1. Micropipetts, 20 µL and 100 µL

2. A measuring cylinder, 500 mL
3. An aspirator and a polypropylene washing bottle, or a microplate washer
4. A microplate mixer
5. A dark box (a dark shelf or drawer will do)
6. A microplate reader (absorbance at 450 nm [A₄₅₀])

PROCEDURE

Deliver negative and positive controls, as well as test sera, in accordance with the layout in the table.

1. *Prepare Washing Solution.* Dilute the condensed washing solution in the kit to 1 : 20 with distilled water. Store it at 2–8 °C before and after use. Be sure that all reagents are warmed up to 15–30 °C before the test.

2. *Negative and Positive Controls.* They are tested in duplicate so as to obtain the mean value. In a lane for the detection of a certain epitope, dispense 50 µL of negative control (good for any epitopes) in each of two wells and 50 µL of the corresponding positive control in other each of two wells.

3. *Delivery of Test Sera to Wells.* To each of four wells (for a test), deliver 50 µL of diluent, and then add 10 µL of the test serum in it (final dilution: 1 : 6).

	I	II	III	IV
1	NC	NC	NC	NC
2	NC	NC	NC	NC
3	PC (m)	PC (k)	PC (s)	PC (u)
4	PC (m)	PC (k)	PC (s)	PC (u)
5	S1	S1	S1	S1
6	S2	S2	S2	S2
7	S3	S3	S3	S3
8	S4	S4	S4	S4

Epitope m
Epitope k
Epitope s
Epitope u

FOR THE DETECTION OF

NC :Negative control
 PC (m) :Positive control for epitope m
 PC (k) :Positive control for epitope k
 PC (s) :Positive control for epitope s
 PC (u) :Positive control for epitope u
 S1–S4 :Test sera (1–4)

Note: A plate can be divided into two halves each containing 12 lanes x 4 wells. Thus, a plate is good for 20 tests when it is used in a single time, and for 8 tests when it is used twice. Unused sections can be spared for a future use, when the plate is contained in an aluminum bag with a desiccant and stored at 2–8 °C.

4. *The First Reaction.* Seal the plate with a plastic adhesive film and shake it at 15–30 °C for 2 hours on a plate mixer at 200–700 rotations per minute (RPM). The reaction is completed, also, by leaving the plate at 15–30 °C for 4 hours.

5. *Washing.* Remove the film from the plate and discard the solution in wells by an aspirator. Fill each well with washing solution (the item 1 in this section) using polypropylene bottle with a narrow-pointed beak, capsize and shake the plate vigorously. Repeat the washing five times. Then, strike the capsized plate against a paper towel to remove any fluid left over in it. (The whole washing procedure can be performed in a microplate washer).

Note: Be sure not to dry out wells of the plate during washing; perform the washing procedure swiftly.

6. *Add Labeled mAb.* To each of the four wells for a test, deliver 50 µL of labeled mAb to epitope m (orange), 50 µL of labeled mAb to epitope k (yellow), 50 µL of labeled mAb to epitope s (blue), and 50 µL of labeled mAb to epitope u (red).

7. *The Second Reaction.* Seal the plate and shake it at 15–30 °C for 2 hours on a plate mixer at 200–700 rotations per minute (RPM). The reaction is completed, also, by leaving the plate at 4 °C for 16–24 hours (overnight).

8. *Washing.* Wash the plate by the procedure shown in the item 5 of this section.

9. *Add Substrate Solution.* Deliver 50 µL of the substrate solution to each well.

10. *Color Development.* Seal the plate, and leave it at 15–30 °C for 30 min in the dark.

11. *Termination of Color Development.* Remove the film from the plate, and deliver 50 µL of stopper solution to each well. Agitate the plate until the developed blue color turns into yellow.

12. *Reading the Absorbancy.* Determine the absorbancy at 450 nm (A_{450}) in a microplate reader. It is necessary to read it within 30 min after stopping the color development.

READING THE RESULTS

1. Determination of the Cut-Off Value for Detecting Each PreS2 Epitope

(1) A_{450} of negative controls should be < 0.1 .

(2) A_{450} of the positive control for each epitope should be > 1.0 .

(3) Calculate the cut-off value: the mean A_{450} value (of two negative controls for each epitope) + 0.1.

(4) *Positive and Negative Results*

Positive sera should have A_{450} values $>$ the cut-off A_{450} value

Negative sera should have A_{450} values $<$ the cut-off A_{450} value

2. *Determination of HBV Genotypes*

Genotypes are determined by the combination of four epitopes (m, k, s and u)

in accordance with the table below.

HBV Genotypes	PreS2 Serotypes	PreS2 Epitopes			
		m	k	s	u
A	su	—	—	+	+
B	m	+	—	—	—
C	ks	—	+	+	—
D (E)	ksu	—	+	+	+
F	k	—	+	—	—

CAUTIONS

(1) Genotypes A, B, C and D are determined in sera with an HBsAg concentration of 20–80 ng/mL, because epitopes m, k, s and u can be detected on HBsAg at this concentration. Since the sensitivity of detecting the epitope k in sera with HBsAg of genotype F is less than 1/20 of that in sera with HBsAg of genotype C or D, sera testing negative for any of the four preS2 epitopes may still have the genotype F, if it contains a sufficient amount of the preS2-product and exhibits epitope b (the common epitope of the preS2 region product). This can be confirmed by applying more amounts of sera for the detection of epitope k. (*see below*)

(2) Sera with low HBsAg concentrations may not test positive for any of the four epitopes. The sensitivity of detection can sometimes be increased by applying 30 µL of the test serum (instead of the regular 10 µL) along with 30 µL of diluent to the well (final dilution: 1 : 2). Avoid applying undiluted sera to wells, lest they should incur false positive reactions.

(3) Genotype E has the same serotype (ksu) as genotype D by this EIA with four mAbs.⁶ Hence, the kit cannot distinguish genotype E from genotype D. HBV of genotype E is found mainly in West Africa; its geographical distribution is much narrower than HBV of genotype D. Taking this into account, the serotype ksu is judged as genotype D by the present EIA kit, allowing for a little possibility for genotype E.

(4) Sera with Abnormal Results. Despite their high HBsAg titers, rare sera can show combinations of the four preS2 epitopes which do not correspond to any of the five major genotypes (A, B, C, D and F), or reveal A₄₅₀ values not comfortably higher than the cut-off value for some epitopes. They may contain HBsAg particles of two or more genotypes, due to double (or multiple) infection with HBV of different genotypes. Or else, they may have mutations in the preS2 region which result in the modification of preS2 epitopes.

(5) As shown in the table, there are some correlation between genotypes and HBsAg subtypes. For HBsAg samples of genotypes A, B, C and D, which have been studied in fairly large numbers in the past, subtypes adr and ayw are restricted to genotype C, while genotype D is associated with HBsAg of subtype ayw exclusively.

HBV Genotypes	HBsAg Subtypes
A	adw, ayw
B	adw, ayw
C	adr, adw, ayr
D	ayw
E	ayw
F	adw

PRECAUTIONS

General

- (1) Reagents should be prewarmed to 15–30 °C before testing.
- (2) Do not mix-use reagents from kit boxes with different lot numbers.
- (3) Adhere strictly to the order of procedure.
- (4) Be sure not to contaminate reagents.
- (5) Wash the equipments and rinse them thoroughly with purified water before use.
- (6) Use a new microtip for every test serum.

Procedures

- (1) Prepare wells for negative and positive controls for each testing.
- (2) Do not rub the plate vigorously or touch the bottom of wells. Be sure not to dry it out.
- (3) Read A_{450} values within 30 min after stopping color development.

Handling

- (1) Sera containing HBsAg are potentially infectious. Exert the maximal caution not to touch test sera with bare hands or let them splash into the eye. Use disposable gloves in handling test sera, and wash your hands thoroughly after completing the test.
- (2) Disinfect test sera, reagents and equipments before disposing them by any of the following methods.
 - a) Immerse them in 0.05% (vol/vol) formalin at 37 °C for more than three days.
 - b) Immerse them in 2% (vol/vol) glutaraldehyde for more than one hour.
 - c) Autoclave them for 20 min at 121 °C.
- (3) Do not let enzyme substrate or stopper solution contact with the skin or mucous

- membranes (eyes, nose and mouth). They are toxic and can cause burns.
- (4) Negative and positive controls, as well as diluent, contain sodium azide (NaN₂). Discard them while water is running for diluting them out.

Others

- (1) Do not use equipments and reagents in the kit for purposes other than the determination of HBV genotypes by serotyping.
- (2) This kit is for research purposes. Do not use it for clinical diagnosis.

STORAGE AND SHELF LIFE

The kit can be used up to 6 months after the production when it is stored at 2–8 °C (*do not freeze the kit*). The expire date is indicated on the box. Negative and positive controls should be taken out from the kit and kept frozen at –20 °C, as soon as the kit is received, and keep them frozen at –20 °C after they are consumed partially.

REFERENCES

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