INSTRUCTION MANUAL
FOR
HBsAg SUBTYPE EIA
EIA based hepatitis B surface antigen subtyping kit with monoclonal antibodies

I. Kit configuration
1. Microplate coated with anti-HBs\textsubscript{a} (96 wells) .............................................. 1 plate
2. HBsAg negative control ........................................................................ 1.0 mL x 1 vial
3. HBsAg positive control (d) .............................................................. 0.5 mL x 1 vial
4. HBsAg positive control (y) .............................................................. 0.5 mL x 1 vial
5. HBsAg positive control (w) .............................................................. 0.5 mL x 1 vial
6. HBsAg positive control (r) .............................................................. 0.5 mL x 1 vial
7. Enzyme labeled monoclonal antibody (d) ...................................... 1.5 mL x 1 vial
8. Enzyme labeled monoclonal antibody (y) ...................................... 1.5 mL x 1 vial
9. Enzyme labeled monoclonal antibody (w) ...................................... 1.5 mL x 1 vial
10. Enzyme labeled monoclonal antibody (r) ...................................... 1.5 mL x 1 vial
11. Enzyme substrate ............................................................................. 10 mL x 1 vial
    (Hydrogen peroxide, 2.5 µL and Tetramethylbenzidine, 5 mg)
12. Reaction stopper .............................................................................. 10 mL x 1 vial
13. Concentrated washing solution ....................................................... 25 mL x 2 vials
14. Plate seal .......................................................................................... 5 sheets

II. Application
Determination of subtypes of hepatitis B surface antigen (HBsAg) in serum or plasma.

III. Principle of assay
This kit has been developed as a reagent for research purposes to detect respective subtypic determinants, \textit{d}, \textit{y}, \textit{w}, and \textit{r}, in hepatitis B surface antigen (HBsAg) positive samples for identification of HBsAg subtypes such as \textit{adw}, \textit{adr}, \textit{ayw} and \textit{ayr}. Detection of subtypic determinants is based on the solid-phase sandwich EIA (Enzyme Immuno Assay). 96 wells of the microplate are coated with monoclonal antibody against the common determinant \textit{a} of HBsAg. HBsAg in samples dispensed to wells is caught on the solid phase and their subtypic determinant, \textit{d}, \textit{y}, \textit{w}, or \textit{r}, is detected by peroxidase-labeled monoclonal antibody against corresponding determinant.

1st reaction

2nd reaction

Revised: January, 1998
IV. Operation

1. Preparation of washing solution.
   Dilute 20 times the concentrated washing solution with distilled water and keep it in a clean bottle at 2 ~ 10°C.

2. Materials required but not supplied with this kit
   1) Micro pipettes, 50 µL and 100 µL.
   2) Pipettes capable of delivering 2 mL and 3 mL, 2 ea.
   3) Incubator capable of controlling temperature at 37°C.
   4) Measuring cylinder, 300 mL or larger, 1 ea.
   5) Aspirator (or plate washer), 1 ea.
   6) Dark box (light-tight cupboard or desk drawer will do), 1 ea.
   7) Dual wavelength microplate reader (main wavelength 450 nm and sub wavelength 630 nm)

3. Determination
   **Make sure to return the kit to 15 ~ 30°C before use.**

1) Addition of samples and the control sera
   Dispense 50 µL each of samples and the control sera to respective wells.
   Following chart shows a recommended pattern of wells assigned to samples and the control sera. Wells can be assigned in any way, however.

   An example of well layout

   **Note:**
   Microplate can be divided in to 12 strips. Strips not used should be kept in an aluminum pouch together with desiccant and stored at 2 ~10°C.

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   B : Blank
   N : Negative Control
   1~21 : Samples
   P-d : Positive Control d
   P-y : Positive Control y
   P-r : Positive Control r
   P-w : Positive Control w

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   N : Negative Control
   1~21 : Samples
   P-d : Positive Control d
   P-y : Positive Control y
   P-r : Positive Control r
   P-w : Positive Control w

2) 1st reaction
   Cover the microplate with the plate seal to prevent evaporation and leave the microplate to stand for 3 hrs at 37°C or 16 ~ 24 hrs at 15 ~ 30°C. Incubation for 16 ~ 24 hrs at 15 ~ 30°C assures higher sensitivity.

3) Washing
Remove the plate seal from the microplate.

Remove samples and microplate contents from all wells with an aspirator. Fill wells with the washing solution, turn the microplate upside down, and shake out the washing solution. Repeat this 5 times.

Hold the microplate upside down and tap it against clean paper towel to thoroughly remove the washing solution from all wells.

**Note:**
While washing the microplate, care should be taken not to dry the microplate well surface. After washing the microplate, immediately follow the following step.

4) **Addition of the labeled antibody (2nd reaction)**

Dispense labeled monoclonal antibody against determinant d, y, w or r to corresponding wells in the following manner.

- Dispense 50 µL each of the labeled monoclonal antibody against:
  - determinant d to wells for detecting determinant d,
  - determinant y to wells for detecting determinant y,
  - determinant w to wells for detecting determinant w, and
  - determinant r to wells for detecting determinant r.

**Note:**
Do not add the labeled monoclonal antibodies to the blank wells.

5) **2nd reaction**

Cover the microplate with the plate seal and incubate for 2 hrs at 37°C.

6) **Washing**

Repeat washing as in 3) above.

7) **Addition of the enzyme substrate**

Add 100 µL of the enzyme substrate to all wells and leave the microplate to stand for 30 min at 15 ~ 30°C in the dark (dark box, light tight cupboard or desk drawer).

8) **Enzyme reaction**

Cover the microplate with the plate seal and incubate at 15 ~ 30°C for 30 min in the dark.

**Note:**
This enzyme substrate contains volatile organic solvent and adversely affects other enzyme reaction (OPD coloring reaction). Do not use this kit under the same environment with other kits using OPD coloring reaction.

9) **Addition of the reaction stopper**

Remove the plate seal and stop color development by adding 100 µL of the reaction stopper to all wells.

10) **Absorbance measurement**

Measure absorbance of each well by a microplate reader (main wavelength 450 nm and sub wavelength 630 nm).

**Note:**
- Adjust the zero point using the blank well.
- Absorbance must be measured within 30 min after stopping color development.

Revised: January, 1998
V. Interpretation of the results

1. Positive and negative determination.
   Calculate the Cut-off value, that is, the absorbance of the Negative Control + 0.200.
   Positive: Absorbance of samples > Cut-off value
   Negative: Absorbance of samples < Cut-off value

2. Determination of HBsAg subtypes
   1) When HBsAg titer of samples is $2^6$ or higher by the reversed passive hemagglutination method (R-PHA), subtypic determinants can be easily detected. When the titer is low, however, their detection sometimes becomes difficult.

   **Note:**
   Avidity of labeled monoclonal antibodies against determinant $d$, $y$, $w$, or $r$ is not alike; that of anti-$r$ and anti-$y$ is higher while that of anti-$d$ and anti-$w$ is lower. Therefore, determinants $d$ and $w$ in samples with low HBsAg titers (lower than $2^6$ by R-PHA with a sensitivity of 3 ng/mL) may not be efficiently determined.

   2) Generally, samples test positive for either determinant $d$ or $y$, as well as for either determinant $w$ or $r$, corresponding to the four major HBsAg subtypes, $adr$, $adw$, $ayw$, or $ayr$. In rare samples, however, more than 3 of $d$, $y$, $w$ and $r$ (compound subtypes) are detectable.

VI. Cautions

1. General cautions
   1) Make sure to return the kit to 15 ~ 30°C before use.
   2) Do not mix reagents of different production lots.
   3) Strictly follow the operational instructions given in this manual.
   4) Avoid contamination of the reagents in this kit.
   5) Materials to be used for the assay must be clean and thoroughly washed in advance with purified water.
   6) Pipette tips must be replaced for each sample.

2. Operational cautions
   1) Measure blank and negative and positive controls for each assay.
   2) Do not scrape or touch the bottom of wells or do not dry the surface of the wells during assaying.
   3) Absorbance must be measured within 30 min after stopping the coloring reaction.

3. Handling cautions
   1) HBsAg positive and negative controls supplied with this kit and samples should be handled as if they were capable of transmitting hepatitis. Wear disposable globes and thoroughly wash hands after assaying.
   2) Before discarding, treat samples, reagents and materials in either of the followings.
      - Immerse in 0.05% formalin for over 72 hrs at 37°C.
      - Immerse in 2% glutaraldehyde solution for over 1 hr.
      - Immerse in sodium hypochlorite solution (more than 0.1%) for over 1 hr.
      - Autoclave for 20 min at 121°C.
   3) Avoid contact of the enzyme substrate and the reaction stopper with skin or...
mucosal membrane. They are toxic and irritable and may cause burn.

4) The enzyme labeled antibodies contain thimerosal. Discarded them with a sufficient amount of running water. Their containers must be washed with a sufficient amount of running water before discarded. The HBsAg positive and negative control sera contain sodium azide and should be washed down with a sufficient volume of water to prevent formation of explosive metal azide.

VII. Storage and shelf life

Kits stored at 2 ~ 10°C are stable for 1 year after the date of manufacture. (Do not freeze). Validity of kits is shown in the package.

VIII. Package

1 kit for 24 tests.

IX. Reference


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