Hepatitis B

anti-HBs (Quantitative)

ANTIBODY TO HEPATITIS B VIRUS SURFACE ANTIGEN ELISA

One-Step Incubation, Double Antigen Sandwich Principle

INSTRUCTIONS FOR USE

INTENDED USE

This anti-HBs ELISA kit is an enzyme linked immunosorbent assay for in vitro quantitative detection of antibodies to hepatitis B virus surface antigen (anti-HBs) in human serum or plasma for clinical purposes and assessing antibody response levels to HBsAg-vaccine.

SUMMARY

Hepatitis B virus (HBV) is an enveloped, double-stranded DNA virus belonging to the Hepadnaviridae family and is recognized as the major cause of blood transmitted hepatitis together with hepatitis C virus (HCV). Infection with HBV induces a spectrum of clinical manifestations ranging from mild, inapparent disease to fulminant hepatitis, severe chronic liver diseases, which in some cases can lead to cirrhosis and carcinoma of the liver. Classification of a hepatitis B infection requires the identification of several serological markers expressed during three phases (incubation, acute and convalescent) of the infection. Now several diagnostic tests are used for screening, clinical diagnosis and management of the disease.

Hepatitis B surface antigen (HBsAg) is an important viral envelope protein, which appears shortly after infection and is a key serological marker for detection and diagnosis of HBV. Clearance during treatment shows recovery and development of neutralizing antibodies (anti-HBs) occurs in 90% of the patients. Due to the introduction of hepatitis B vaccination programs, the detection of anti-HBs has become important method for monitoring of recipients upon vaccination with synthetic and natural HBsAg. The absence of anti-HBs indicates susceptibility to HBV infection. For this, screening for anti-HBs in high-risk populations is recommended for identifying individuals who may benefit from vaccination.

PRINCIPLE OF THE ASSAY

For detection of anti-HBs, this kit uses antigen “sandwich” ELISA method where polystyrene microwell strips are pre-coated with recombinant HBsAg. Patient’s serum or plasma sample is added to the microwells together with a second HBsAg conjugated to Horseradish Peroxidase (HRP-Conjugate). In case of presence of anti-HBs in the sample, the pre-coated and conjugated antigens will be bound to the two variable domains of the antibody and during incubation, the specific immunocomplex formed is captured on the solid phase. After washing to remove sample and unbound HRP-Conjugates, Chromogen solutions containing Tetramethylbenzidine (TMB) and urea peroxide are added to the wells. In presence of the antigen-antibody-antigen(HRP) “sandwich” complex, the colorless Chromogens are hydrolyzed by the bound HRP-Conjugate to a blue-colored product. The blue color turns yellow after stopping the reaction with sulfuric acid. The amount of color intensity can be measured and is proportional to the amount of antibody captured in the wells, and to the sample respectively.

Assay principle scheme: Double antigen sandwich ELISA

Ag(p) +Ab(s) + (Ag)ENZ→ [Ag(p)–Ab(s)–(Ag)ENZ] →Blue→Yellow
Ag(p) +(Ag) ENZ→ [Ag (p)] →No Color
Incubation Immobilized Complex Coloring
60 min. 15min.

Ag(p)–pre-coated HBsAg;
Ab(s)– anti-HBs in sample; vehicle
(Ag)ENZ–HRP conjugated HBsAg;

COMPONENTS

96 tests

- **MICROWELL PLATE** 1plate

Blank microwell strips fixed on a white strip holder. The plate is sealed in aluminium pouch with desiccant.
8×12/12×8-well strips per plate. Each well contains recombinant HBsAg. The microwell strips can be broken to be used separately.
Place unused wells or strips in the plastic sealable storage bag together with the desiccant and return to 2~8℃.

- **CALIBRATION CURVE STANDARDS** 6vials

Yellowish liquids filled in a vial with green screw cap. The kit contains the following standards:
0 mIU, 10 mIU, 20 mIU, 40 mIU, 80mIU, 160mIU/ml
500µl each.
anti-HBs diluted in protein-stabilized buffer.
Preservative: ProClin 300.

- **HRP-CONJUGATE REAGENT** 1vial

Red-colored liquid filled in a white vial with red screw cap.
6.5ml per vial.
Horseradish peroxidase-conjugated HBsAg. Ready to use as supplied. Once open, stable for one month at 2-8℃.

- **STOCK WASH BUFFER** 1bottle

DILUTE BEFORE USE
Colorless liquid filled in a clear bottle with white screw cap.
30ml per bottle.
PH 7.4 20 × PBS (Contains Tween-20 as detergent). The concentration must be diluted **1 to 20** with distilled/deionized water before use. Once diluted, stable for one week at room temperature, or for two weeks when stored at 2-8℃.

- **CHROMOGEN SOLUTION A** 1vial

Colorless liquid filled in a white vial with green screw cap.
7ml per vial.
Urea peroxide solution.
Ready to use as supplied.
Once open, stable for one month at 2-8℃.

- **CHROMOGEN SOLUTION B** 1vial

Colorless liquid filled in a black vial with black screw cap.
TMB solution(Tetramethyl benzidine dissolved in citric acid).
7ml per vial.
Ready to use as supplied.
Once open, stable for one month at 2-8℃.

- **STOP SOLUTION** 1vial

Colorless liquid filled in a white vial with white screw cap.
7 ml per vial.
Diluted sulfuric acid solution (2.0M H_2SO_4).
Ready to use as supplied.

- **PLASTIC SEALABLE BAG** 1unit

For enclosing the strips not in use.

- **CARDBOARD PLATE COVER** 1sheet

To cover the plates during incubation and prevent evaporation or contamination of the wells.

- **PACKAGE INSERTS** 1copy

ADDITIONAL MATERIALS AND INSTRUMENTS REQUIRED BUT NOT PROVIDED

1. Freshly distilled or deionized water.
2. Disposable gloves and timer.
3. Appropriate waste containers for potentially contaminated materials.
4. Disposable V-shaped troughs.
5. Dispensing system and/or pipette (single or multichannel), disposable pipette tips.
6. Absorbent tissue or clean towel.
7. Dry incubator or water bath, 37±0.5℃.
8. Microshaker for dissolving and mixing conjugate with samples.
9. Microwell plate reader, single wavelength 450nm or dual wavelength 450nm and 630nm.
10. Microwell aspiration/wash system.

SPECIMEN COLLECTION，TRANSPORTATION AND STORAGE

1. **Sample Collection:** Either fresh serum or plasma samples can be used for this assay. Blood collected by venipuncture should be allowed to clot naturally and completely – the serum/plasma must be separated from the clot as early as possible as to avoid hemolysis of the RBC. Care should be taken to ensure that the serum samples are clear and not contaminated by microorganisms. Any visible particulate matters in the sample should be removed by centrifugation at 3000 RPM for at least 20 minutes at room temperature, or by filtration on 0.22u filters. Plasma samples collected into EDTA, sodium citrate or heparin may be tested, but highly lipaemic, icteric, or hemolized samples should not be used as they could give erroneous results in the assay. Do not heat inactivate samples. This can cause sample deterioration.
2. **Transportation and Storage:** Store samples at 2-8℃. Samples not required for assaying within 3 days should be stored frozen (-20℃ or lower).Multiple freeze-thaw cycles should be avoided. For shipment, samples should be packaged and labeled in accordance with the existing local and international regulations for transport of clinical samples and ethological agents.

SPECIAL INSTRUCTIONS FOR WASHING

1. A good washing procedure is essential to obtain correct and precise analytical data.
2. It is therefore recommended to use a good quality ELISA microplate washer, maintained at the best level of washing performances. In general, no less than 5 automatic washing cycles with dispensing of 350-400µl/well, are sufficient to avoid false positive reactions and high background (all wells turn yellow).
3. To avoid cross-contaminations of the plate with sample or HRP-conjugate, after incubation do not discard the content of the wells, but allow the plate washer to aspirate it automatically.
4. Anyway, we recommend calibrating the washing system on the kit itself in order to match the declared analytical performances. Assure that the microplate washer's liquid dispensing channels are not blocked or contaminated, and sufficient volume of Wash buffer is dispensed each time into the wells.
5. In case of manual washing, we suggest to perform at least 5cycles, dispensing 350-400µl/well and aspirating the liquid for 5times. If poor results (high background) are observed, increase the washing cycles or soaking time per well.
6. In any case, the liquid aspirated out the strips should be treated with a sodium hypochlorite solution(final concentration of 2.5%) for 24 hours, before liquids are disposed in an appropriate way.
7. The concentrated Washing solution should be diluted **1 to 20** before use. For one plate, mix 30 ml of the concentrate with 570ml of water for a final volume of 600ml diluted Wash Buffer. If less than a whole plate is used, prepare the proportional volume of solution.

STORAGE AND STABILITY

The components of the kit will remain stable through the expiration date indicated on the label and package when stored between 2-8 ℃; **do not freeze**. To assure maximum performance of this anti-HBs quantitative ELISA kit, during storage protect the reagents from contamination with microorganism or chemicals.

PRECAUTIONS AND SAFETY

This kit is intended **FOR IN VITRO USE ONLY** IVD

FOR PROFESSIONAL USE ONLY

The ELISA assay is a time and temperature sensitive method. To avoid incorrect result, strictly follow the test procedure steps and do not modify them.

1. Do not exchange reagents from different lots, or use reagents from other commercially available kits. The components of the kit are precisely matched as to achieve optimal performance during testing.
2. Make sure that all reagents are within the validity indicated on the kit box and are of the same lot. Never use reagents beyond the expiry date stated on reagents labels or on the kit box.
3. **CAUTION - CRITICAL STEP:** Allow the reagents and samples to stabilize at room temperature(18-30℃) before use. Shake reagent gently before, and return to 2-8℃ immediately after use.
4. Use only sufficient volume of sample as indicated in the procedure steps. Failure to do so, may cause in low sensitivity of the assay.
5. Do not touch the bottom exterior of the wells; fingerprints or scratches may interfere with microwell reading.
6. When reading the results, ensure that the plate bottom is dry and there are no air-bubbles inside the wells.
7. Never allow the microplate wells to dry after the washing step. Immediately proceed to the next step. Avoid the formation of air-bubbles when adding the reagents.
8. Avoid assay steps long time interruptions. Assure same working conditions for all wells.
9. Calibrate the pipette frequently to assure the accuracy of samples/reagents dispensing. Always use different disposal pipette tips for each specimen and reagents as to avoid cross-contaminations. Never pipette solutions by mouth.

10. The use of automatic pipettes is recommended.
11. Assure that the incubation temperature is 37℃ inside the incubator.
12. When adding samples, avoid touching the well's bottom with the pipette tip.
13. When reading the results with a plate reader, it is recommended to determine the absorbance at 450nm or at 450nm with reference at 630nm.
14. All specimens from human origin should be considered as potentially infectious.
15. Materials from human origin may have been used in the kit. These materials have been tested with tests kits with accepted performance and found negative for antibodies to HIV ½, HCV, TP and HBsAg. However, there is no analytical method that can assure that infectious agents in the specimens or reagents are completely absent. Therefore, handle reagents and specimens with extreme caution as if capable of transmitting infectious diseases. Strict adherence to GLP (Good Laboratory Practice) regulations can ensure the personal safety. Never eat, drink, smoke, or apply cosmetics in the assay laboratory.
16. Bovine derived sera may have been used in this kit. Bovine serum albumin (BSA) and fetal calf sera (FCS) are derived from animals from BSE/TSE free-geographical areas.
17. The pipette tips, vials, strips and sample containers should be collected and autoclaved for 1hour at 121℃ or treated with 10% sodium hypochlorite for 30minutes to decontaminate before any further steps for disposal.
18. The Stop solution (2M H_2SO_4) is a strong acid. Corrosive. Use it with appropriate care. Wipe up spills immediately or wash with water if come into contact with the skin or eyes. ProClin 300 used as a preservative can cause sensation of the skin.
19. The enzymatic activity of the HRP-conjugate might be affected from dust, reactive chemical, and substances like sodium hypochlorite, acids, alkalins etc. Do not perform the assay in the presence of such substances.
20. Materials Safety Data Sheet (MSDS) available upon request.
21. If using fully automated microplate processing system, during incubation, do not cover the plates with the plate cover. The tapping out of the remainders inside the plate after washing, can also be omitted.

ASSAY PROCEDURE

Step1 **Reagents preparation:** Allow the reagents and samples to reach room temperature (18-30℃) for at least 15-30minutes. Check the Wash buffer concentrate for the presence of salt crystals. If crystals have formed in the solution, resolubilize by warming at 37℃ until crystals dissolve. Dilute the stock wash Buffer **1 to 20** with distilled or deionized water. Use only clean vessels to dilute the buffer.

Step2 **Numbering Wells:** Set the strips needed in strip-holder and number sufficient number of wells including six calibration curve standards wells **(e.g. B1-G1; H1-E2)** and one Blank (**e.g. A1**, neither samples nor HRP-Conjugate should be added into the Blank well). If the results will be determined by using dual wavelength plate reader, the requirement for use of Blank well could be omitted. Use only number of strips required for the test. Run the standards in duplicates.

Step3 **Adding Sample:** Add **50µl** of Calibration curve standards and **50µl** specimen into their respective wells. **Note: Use a separate disposal pipette tip for each specimen as to avoid cross-contamination.**

Step4 **Adding HRP-Conjugate:** Add **50µl** of HRP-Conjugate Reagent into each well except into the Blank and mix gently. **Never add HRP-Conjugate to the Blank well.**

Step5 **Incubating:** Cover the plate with the plate cover and incubate for **60minutes at 37°C**. It is recommended to use thermostat-controlled water tank as to assure the temperature stability and humidity during the incubation. If dry incubator is used, do not open the door frequently.

Step6 **Washing:** At the end of the incubation, remove and discard the plate sealer. Wash each well **5times** with diluted Wash buffer. Each time, allow the microwells to soak for 30-60 seconds. After the final washing cycle, turn the plate onto blotting paper or clean towel, and tap it to remove any remainders.

Step7 **Coloring:** Dispense **50µl** of Chromogen A and **50µl** Chromogen B solution into each well including the **Blank** and mix by tapping the plate gently. Incubate the plate at **37℃ for 15minutes avoiding light**. The enzymatic reaction between the Chromogen A/B solutions and the HRP-Conjugate produces blue color in Calibration curve standards wells (except for 0mIU/ml) and in anti-HBs positive sample wells.

Step8 **Stopping Reaction:** Using a multichannel pipette or manually add **50µl** Stop Solution into each well and mix gently. The blue color will turn yellow after stopping the reaction.

Step9 **Measuring the Absorbance:** Calibrate the plate reader with the Blank well and read the absorbance at **450nm**. If a dual filter instrument is used, set the reference wavelength at **630nm**. Calculate the results (**Note:** read the absorbance within **10minutes** after stopping the reaction) .

INTERPRETATION OF RESULTS AND QUALITY CONTROL

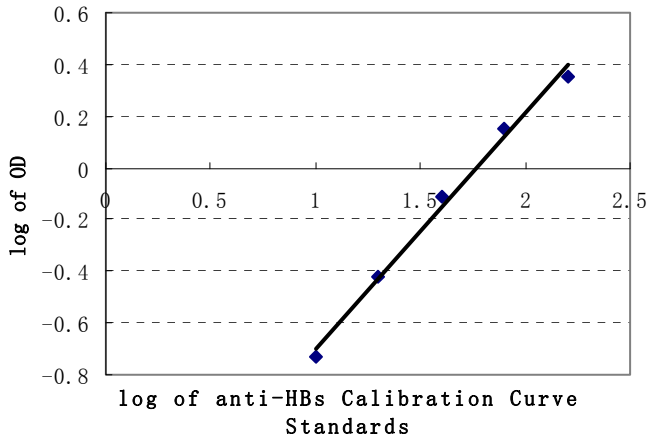
If the results reading is based on single filter plate reader, the results should be calculated by subtracting the Blank well OD value from the print report values of samples and controls. In case the reading is based on dual filter plate reader, do not subtract the Blank well OD from the print report values of samples and controls.

1. Record the absorbance (OD) obtained from the print report of the microplate reader.
2. Plot the absorbance (log-OD) for each duplicate calibration standard on the Y (logarithmic ordinate) versus the corresponding anti-HBs concentration (log-mIU/ml) on the X (logarithmic abscissa) on double-logarithmic paper (do not average the duplicates of the calibration standards before plotting).
3. Draw the standard curve through the plotted points (best-fit).
4. To determine the concentration of anti-HBs for an unknown, locate the absorbance (OD) for each unknown on the Y-axis of the graph, find the intersecting point on the standard curve, and read the concentration (log-ng/ml) from the X-axis of the graph. Calculate the concentration of the unknown in ng/ml.

Example of a Standard Curve:
For illustration purpose only- the average values are given only: (*0mIU/ml=Negative Samples or Negative Control).

Standards	log mIU/ml	Mean OD	log OD
10mIU/ml	1	0.186	-0.728
20mIU/ml	1.30103	0.380	-0.4202
40mIU/ml	1.60206	0.770	-0.1135
80mIU/ml	1.90309	1.427	0.1544
160mIU/ml	2.20412	2.249	0.3521

Example of anti-HBs Standard Curve



Quality control range:

The test results are valid if the Quality Control criteria are verified. It is recommended that each laboratory must establish appropriate quality control system with quality control material similar to or identical with the patient sample being analyzed.

1. The OD value of the Blank well, which contains only Chromogens and Stop solution, is less than 0.080 at 450nm.
2. The OD value of 0mIU/ml standard must be less than 0.100 at 450/630nm or at 450nm after blanking.
3. The OD value of the 160mIU/ml standard must be higher than 1.500 at 450/630nm or at 450nm after blanking.

TEST PERFORMANCE AND EXPECTED RESULTS

Analytical Endpoint Sensitivity: (lower detection limit): In the follow-up of vaccinated individuals the value of 20 WHO mIU/ml is the minimum concentration at witch the recipient is considered protected. This anti-HBs ELISA kit shows sensitivity of 5mIU/ml.

Clinical Sensitivity: The performance characteristics of this assay were evaluated by a panel of samples obtained from 600 individuals receiving HBV vaccines in which the titers of anti-HBs were evaluated in a direct comparison with another commercially available anti-HBs ELISA kit. From this group, 594 individuals showed antibody titers higher than 10mIU, which was confirmed with the reference anti-HBs ELISA kit. In another group of 220 individuals with confirmed hepatitis B vaccination history, 220 of the tested samples showed antibody titer higher than 10mIU. From this study, overall agreement of 100% was obtained between this anti-HBs ELISA kit and the reference test in linear regression analysis.

In a panel of 240 samples obtained from early recovery hepatitis B patients (confirmed HBsAg -, anti-HBc+ and anti-HBs+), sensitivity of 100% was calculated in comparison with the reference test.

Specificity: > 99% calculated by a panel of samples obtained from 500 healthy individuals with confirmed levels of anti-HBs less than 10 mIU/ml.

No cross reactivity observed when testing samples from patients infected with HAV, HCV, HIV, CMV, and TP. No interference from elevated levels of rheumatoid factors up to 2000U/ml. No high dose hook effect up to 150000mIU/ml observed during clinical testing. The kit performance characteristics are unaffected from elevated concentrations of bilirubin, hemoglobin, and triolein. Frozen specimens have been tested to check for interferences due to collection and storage.

Recovery:

HBsAb added mIU/ml	HBsAb measured mIU/ml	Recovery %
0mIU/ml	-	-
20mIU/ml	19.6mIU/ml	98
76mIU/ml	75.0mIU/ml	98.68
94mIU/ml	93.7mIU/ml	99.68
130mIU/ml	149mIU/ml	114
190mIU/ml	185mIU/ml	97.36

Reproducibility		Within Run	Between Run
Standards	Test	CV%	CV%
*0mIU/ml	10	14	14
10mIU/ml	10	7.7	8.1
20mIU/ml	10	7	7.5
40mIU/ml	10	6	7.3
80mIU/ml	10	4.5	4.68
160mIU/ml	10	3.9	4.2

*0mIU/ml=Negative Samples or Negative Control.

LIMITATIONS

1. Non- repeatable reactive results may be obtained with any ELISA test due to the general characteristics of this method. Any positive result must be interpreted in conjunction with the patient clinical information and other laboratory results.
2. If, after retesting of the initially reactive samples, the assay results are negative , these samples should be considered as non-repeatable (false positive) and interpreted as negative. As with many very sensitive ELISA assays, false positive results can occur due to the several reasons, most of which are related but not limited to inadequate washing step.
3. Common sources for mistakes: kits beyond the expiry date, bad washing procedures, contaminated reagents, incorrect assay procedure steps, insufficient aspiration during washing, failure to add samples or reagents, equipment, timing, volumes, sample nature and quality.
4. The prevalence of the marker will affect the assay's predictive values.
5. In some cases, very strong immunological response after vaccination can be observed due to the vaccine biological characteristics. High concentrations of antibodies beyond the standard curve measurement range (>160mIU/ml) can be diluted and retested. Samples may not show linear properties after dilution as the same way as the materials

used for the standards. This phenomenon is frequently observed when samples are tested for antibodies.

6. Samples tested using assay from different manufacturer can give similar quantitative results but some samples can give discrepancies due to the antibodies diversity and the antigenic properties of HBsAg used in the assay.
7. This kit is intended ONLY for testing of individual serum or plasma samples. Do not use it for testing of cadaver samples, saliva, urine or other body fluids, or pooled (mixed) blood.

INDICATIONS OF INSTABILITY OR DETERIORATION OF THE REAGENTS

1. Values of the Positive or Negative controls ,which are out of the indicated Quality control range, are indicator of possible deterioration of the reagents and/or operator or equipment errors. In such case, the results should be considered as invalid and the samples must be retested. In case of constant erroneous results classified as due to deterioration or instability of the reagents, immediately substitute the reagents with new ones.
2. If after mixing of the Chromogen A and B solutions into the wells, the, the color of the mixture turns blue within few minutes, do not continue carrying out the testing and replace the reagents with fresh ones.

VALIDITY

Please do not use this kit beyond the expiry date indicated on the kit box and reagent labels.

REFERENCES:

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4. Jilg, W., et al. (1990). Hepatitis B–vaccination strategy for booster doses in high-risk population groups. Progress in Hepatitis B Immunisation. P. Coursaget, M.J. Tong eds., Colloque INSERM. 194, 419.
5. Engvall E. and Perlmann P. J.Immunochemistry, 8, 871-874, 1971.
6. Engvall E. and Perlmann P. J.Immunol. 109, 129-135, 1971.

Example of controls/samples dispensing scheme

	1	2	3	4	5	6	7	…	…	12
A	Blank	2								
B	1	3								
C	2	4								
D	3	5								
E	4	6								
F	5	S1								
G	6	S2								
H	1	S3								

1…6 (0 mIU, 10 mIU, 20 mIU, 40 mIU, 80mIU, 160mIU/ml HBsAb standards)

SUMMARY OF THE ASSAY PROCEDURE:	
Add Sample/Calibration Curve Standards	**50µl**
Add HRP-Conjugate	**50µl**
Incubate	**60minutes**
Wash	**5times**
Coloring	**50µl A + 50µl B**
Incubate	**15minutes**
Stop the reaction	**50µl stop solution**
Read the absorbance	**450 nm or450/630 nm**

SUMMARY OF THE MAJOR COMPONENTS OF THE KIT:	
Microwell plate	**One/ 96 wells**
Anti-HBs Standards	**Six/ 0.5ml each**
HRP-Conjugate	**One/ 6.5ml**
Wash Buffer	**One/ 30ml**
Chromogen A/B/ Stop Solution	**One each/ 7ml**
Note: the components of individual kits are not interchangeable	