HBsAg
ELISA

Enzyme-linked immunosorbent assay (ELISA) for the detection of the Hepatitis B virus surface antigen (HBsAg)

SUMMARY

Hepatitis B is a viral disease of the liver caused by the hepatitis B virus (HBV). This disease may be asymptomatic or an acute or chronic process. In severe cases it can lead to liver cirrhosis and primary hepatocellular carcinoma. It is primarily transmitted by parenteral, percutaneous or sexual contact. Perinatal and horizontal transmission has also been observed.

HBV consists of a nucleocapsid containing DNA associated to core proteins and a capsid whose main component is a protein known as surface antigen (HBsAg). HBsAg usually appears 6 weeks after exposure to HBV and persists for 4-14 weeks. It is present during the incubation period, before the onset of the clinical disease and it can be detected in blood 2 to 8 weeks before the onset of jaundice or biochemical evidence of liver dysfunction. Thus, HBsAg is a first indicator of HBV infection. Chronic hepatitis B is defined as presence of HBsAg in blood for more than 6 months.

HBsAg detection is important for the diagnosis of acute and chronic hepatitis, carrier control in blood banks, dialysis and transplanted patient units, pregnant women and for control of blood preparations and derivatives intended for transfusion.

PRINCIPLE

HBsAg ELISA is a sandwich direct enzyme immunoassay method. The wells of the microtitration plate are coated with anti-HBs guinea pig antibody (anti-HBsAg) acting as a capture antibody. The sample is incubated in one well. If it contains HBsAg, it will form a complex with the antibody bound to the plate. The unbound material is removed by washing. Then, the anti-HBs goat antibody conjugated to peroxidase is added, which will bind to the antibody-antigen preformed complex. The unbound conjugate is removed by washing. Subsequently, a solution containing tetramethylbenzidine and hydrogen peroxide is added. In cases in which HBsAg is present in the sample, a light-blue color is developed which becomes yellow when the reaction is stopped with sulfuric acid.

PROVIDED REAGENTS

Coated microtitration plate: microtitration plate with cutout strips and 96 wells coated with anti-HBs guinea pig antibody.
Concentrated Conjugate: goat anti-HBs antibody conjugated to peroxidase (51x). Red color.
Conjugate Diluent: Tris buffer with proteins and preservatives.
TMB: 36 mM tetramethylbenzidine solution (TMB) in dimethylsulfoxide (DMSO) (100x).
TMB diluent: 40 mM citrate buffer and 1.27 mM hydrogen peroxide, pH 4,3.
Stopper: 2 N sulfuric acid.
Concentrated Wash Buffer: saline buffer with surfactant (25x). Green color.
Positive Control: serum containing inactivated HBsAg and preservatives. Orange color.
Negative Control: serum with preservatives. Yellow color.

NON-PROVIDED REAGENTS

Distilled or deionized water.

REQUIRED MATERIAL (non-provided)

- Micropipettes for measuring stated volumes
- Disposable tips
- Volumetric material to prepare stated dilutions
- 37°C incubator
- Absorbent paper
- Disposable gloves
- Timer or stopwatch
- Sodium hypochlorite
- Microtitration plate wash system (manual or automatic)
- Spectrophotometer for microtitration plate reading

WARNINGS

- The reagents are for “in vitro” diagnostic use.
- All patient samples should be handled as capable of transmitting infection.
-- The control sera have been tested for antibodies to Human Immunodeficiency Virus (HIV) and Hepatitis C Virus (HCV), and found to be non-reactive. However, they should be handled as potentially infectious material.
- All materials used to perform the test must treated to ensure the inactivation of pathogenic agents. The recommended method is autoclaving for one hour at 121°C. Liquid waste may be disinfected with sodium hypochlorite (5% final concentration) for at least 60 minutes.
- Avoid contact of strips with hypochlorite fumes from biohazards disposal containers or other sources, since hypochlorite affects the reaction.
- Avoid liquid spilling and aerosol formation.
- Do not use the reagents after the expiration date.
- Do not exchange reagents from different lots and do not modify assay procedures.
- Do not use reagents from other origin.
- Avoid touching the walls of the wells with the tips.
- Avoid using metal objects that may be in contact with the reagents.

- The microtitration plates should be incubated in incubator. Do not use water bath. Do not open the incubator during this process.
- Avoid contact of the sulfuric acid (Stopper) and DMSO (TMB) with the skin, mucous membranes and eyes. If this happens, rinse with plenty of water. H315+H320: Causes skin and eye irritation. H314: Causes severe skin burns and eye damage. P262: Do not get in eyes, on skin, or on clothing. P305 + P351 + P338: IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. P302 + P352: IF ON SKIN: Wash with plenty of soap and water. P280: Wear protective gloves/ protective clothing/eye protection/face protection.
- Do not pipette by mouth. Use disposable gloves and eye protection during handling of samples and reagents.
- TMB is sensitive to light. Keep the bottle capped when unused.
- All reagents and samples should be discarded according to the local regulations in force.

REAGENT PREPARATION

Wash Buffer: constituents of the concentrated reagent may precipitate at low temperature. In such case, bring the solution to 37ºC until complete dissolution. To obtain a ready-to-use wash buffer (1x), dilute 1 part Concentrated Wash Buffer (25x) with 24 parts distilled or deionized water. Example: 20 ml with 480 ml for one microtitration plate.

Conjugate: to obtain a ready-to-use conjugate (1x), dilute 1 part Concentrated Conjugate + 50 parts Conjugate Diluent according to the following table:

Nº of wells	Concentrated Conjugate	Conjugate Diluent
8	20 ul	1 ml
16	40 ul	2 ml
24	60 ul	3 ml
32	80 ul	4 ml
96	240 ul	12 ml

Substrate: to obtain a ready-to-use substrate (1x), dilute 1 part TMB (100x) + 100 parts TMB Diluent (see the following table). Concentrated TMB is dissolved in DMSO. Since the melting temperature of DMSO is 18 ºC, TMB must reach room temperature (20-25 ºC) and be well homogenized before use.

Nº of wells	TMB	TMB Diluent
8	10 ul	1 ml
16	20 ul	2 ml
24	30 ul	3 ml
32	40 ul	4 ml
96	120 ul	12 ml

Stopper, Negative and Positive Controls: ready to use.

STABILITY AND STORAGE INSTRUCTIONS

Provided Reagents are stable in refrigerator (2-10ºC) until expiration date stated on the box. Do not freeze.

Concentrated Wash Buffer and Stopper: they may be stored at room temperature (2-25ºC).

Wash Buffer (1x): store in sealed container. It is stable for 3 months at room temperature (2-25ºC).

Conjugate (1x): it is stable for 7 days at 2-10 ºC, and for 6 hours at 20-25 ºC.

Coated microtitration plate: do not open the pouch until performing the test and until it has reached room temperature. Otherwise, the well strip surface could get moistened. Unused well strips should be kept in the pouch with the desiccant, sealed stored at 2-10 ºC. Test strips stored in this manner are stable for 4 months if it does not exceed the date printed on the pouch label.

SAMPLE

Serum or plasma

a) Sample collection: obtain in the usual way.

b) Additives: not required for serum. Serum collected in tubes containing coagulation accelerator and separator gel may be used. Employ plasma collected using EDTA, heparin or sodium citrate as anticoagulants.

c) Known interfering substances: no interference has been observed with bilirubin up to 25 mg/dl, ascorbic acid up to 50 mg/dl, triglycerides up to 1200 mg/dl, hemoglobin up to 300 mg/dl. Samples containing particles should be clarified by centrifugation.

d) Stability and storage instructions: sample should be stored at 2-10 ºC up to 3 days. If extended storage periods are required, samples should be frozen at -20 ºC (or less). Samples should not be repeatedly frozen and thawed. This may lead to erroneous results. In case of using frozen samples, they should be homogenized and centrifuged before use.

Heat inactivation may affect the result.

Do not use samples with microbial contamination.

If samples are to be transported, they should be packaged according to local regulations for biohazard material shipment.

TEST PROCEDURE

1- Bring the reagents and samples to room temperature before starting the test.

2- Prepare the necessary volume of Wash Buffer (1x).

3- Place the required wells in the strip holder for the number of determinations to be used, including 2 wells for Positive Control (PC) and 3 for Negative Control (NC).

4- Dispense the Sample (S) and the controls according to the following scheme:

	S	PC	NC
Positive Control	-	100 ul	-
Negative Control	-	-	100 ul
Sample	100 ul	-	-

Controls or samples dispensing to the wells may be verified visually or by spectrophotometric reading (at 410/450 nm).

5- To avoid evaporation, cover the plate with the provided adhesive tape and incubate for 60 ± 2 minutes at 37 ± 1ºC. At the same time, prepare the conjugate (see table in REAGENTS PREPARATION).

6- The liquid from each well must be thoroughly removed after incubation. Wash 5 times according to washing instructions (see WASHING PROCEDURE).
7- Add the Conjugate:

Conjugate	100 ul	100 ul	100 ul

To avoid evaporation cover the microplate with adhesive tape.
8- Incubate for 30 ± 1 minute at 37 ± 1ºC. At the same time, prepare the substrate (see table in REAGENTS PREPARATION).
9- Wash 5 times according to the washing instructions.
10- Dispense the Substrate.

Substrate	100 ul	100 ul	100 ul

11- Incubate for 30 ± 2 minutes at room temperature (18-25 ºC), protecting from light.
12- Add the Stopper:

Stopper	100 ul	100 ul	100 ul

13- After 5 minutes, read absorbance in spectrophotometer bichromatically at 450/620-650 nm, or monochromatically at 450 nm.
Note: bichromatic reading is recommended. In case the reading is monochromatic, perform a reagent blank that will have to be subtracted from all sample values.

STABILITY OF FINAL REACTION
Reaction color is stable for 5-30 minutes. Thus, results should be read within this period.

WASHING PROCEDURE
Remove the liquid from the wells by aspiration or inversion.
The wells are washed with 350 ul Wash Buffer. When filling the wells make sure not to spill. The wash solution should be in contact with the wells for 30 to 60 seconds. In case washing is automatically performed, it is advisable to perform a double aspiration after each wash. Ensure that after the last wash no residual liquid is left. Consequently, perform a double aspiration to remove excess buffer. If it persists after such procedure, invert the plate on absorbent paper and tap several times, otherwise erroneous results may be obtained.

Note: the washing procedure is crucial for the test result. If excess wash buffer remains in the wells or if the wells are not completely filled, erroneous results may be obtained.
Do not let the wells air dry during the procedure. Automatic washers should be rinsed with distilled or deionized water at the end of the day to avoid obstructions due to the presence of salt in the wash buffer.

SUMMARY OF THE PROCEDURE

STAGE	PROCEDURE	WARNINGS/OBSERVATIONS
Dilution	Prepare Wash solution (1x)	Dissolve salt crystals
Samples	Add 100 ul S, PC and NC	
Incubation	Cover the wells and incubate for 60 ± 2 minutes at 37 ± 1ºC	In incubator
Washing step	Wash each well with 350 ul diluted Wash Buffer (5 times)	Time of contact of the Wash solution from 30 to 60 seconds. Completely remove the residual liquid from the wells
Dilution	Conjugate preparation(1x)	During incubation with the sample, dilute the Concentrated Conjugate (51x)
Conjugate	Add 100 ul Conjugate (1x)	
Incubation	Cover the wells and incubate for 30 ± 1 minutes at 37 ± 1ºC	In incubator
Washing step	The same as above	
Dilution	Substrate preparation (1x)	During incubation with the Conjugate, dilute TMB (100x)
Substrate	Add 100 ul Substrate	Avoid contact with oxidizing agents. Avoid light exposure
Incubation	30 ± 2 minutes at 18-25ºC	Keep the wells protected from light
Stop	Add 100 ul Stopper	
Reading	Read in spectrophotometer	Read within 5 to 30 minutes

ASSAY VALIDATION CRITERIA

The assay is considered valid if the following conditions are simultaneously met:

1- he mean absorbance of the Negative Controls should be less than or equal to 0.050.
Example:
Reading 1 = 0.025, Reading 2 = 0.028, Reading 3 = 0.022
Mean = (0.025 + 0.028 + 0.022) / 3= 0.025

2- For calculations remove any Negative Control with absorbance greater than 0.050.

3- If any Negative Control has been removed, recalculate the Negative Control mean. An assay is valid when at least two of the Negative Controls are accepted.

4- The absorbance mean of the Positive Controls should be greater than 1.000.
Example:
Reading 1 = 1.504, Reading 2 = 1.496
Mean = (1.504 + 1.496) / 2= 1.500

5- The absorbance mean difference of the Positive and Negative Controls should be greater or equal to 0.950.

In case one of the above conditions is not met, repeat the assay.
Remember that the obtained readings will depend on the sensitivity of the instrument used.

INTERPRETATION OF RESULTS

The presence or absence of HBsAg antigen is determined associating the sample absorbance with the Cut-off value.

Cut-off = NC + 0.060

NC: absorbance mean of the Negative Control

Example: 0.025 + 0.060 = 0.085

Non-reactive samples: samples with absorbances lower than the Cut-off value.
Reactive samples: samples with absorbances greater or equal to the Cut-off value.
All samples initially reactive should be repeated by duplicate. If one or both repetitions are reactive, it should be considered reactive.
A sample initially reactive may be non-reactive in both repetitions. This may be due to:
- Cross contamination of a non-reactive well with a reactive sample.
- Sample contamination during dispensation, lack of precision in sample, conjugate and/or Substrate dispensation into the well.
- Tip re-utilization.
- Well contamination with hypochlorite or other oxidizing agents.

In certain cases a non-reactive sample may produce a falsely reactive reaction, both in the initial analysis as in its repetitions. Some probable causes of this effect may be:
- Sample contamination during collection, processing or storage.
- Presence of interfering substances, such as autoantibodies, drugs, etc.
- Ineffective dispensation and/or aspiration of the wash solution (obstructed system).

PROCEDURE LIMITATIONS

See Known interfering substances under SAMPLE.
Do not use pooled serum or plasmas.
Do not use other body fluids such as saliva, cerebrospinal fluid or urine.
A non-reactive result does not exclude the possibility of HBV infection since the antigen can be found at a lower concentration to the detection limits.
If a sample is repeatedly reactive confirmatory testing and other serological markers of hepatitis B should be performed to confirm HBV infection.

SPECIFIC PERFORMANCE FEATURES

Clinical Sensitivity

In a study of 122 samples with HBsAg infection, confirmed by different methods, all the samples were found repeatedly reactive by the HBsAg ELISA kit.

Sensitivity in performance panels

Various international commercial panels were tested obtaining the following results:

Panel	Reactive samples	Detected samples
PHA105	14	13
PHA106M	12	11
PHA204	23	22
PHA205	23	23
PHA206	23	20

PHA105, and PHA106M (HBsAg Low Titer Performance Panels, BBI, USA).
PHA204, PHA205, and PHA206 (HBsAg Mixed Titer Performance Panels, BBI, USA).

Sensitivity in Seroconversion Panels

The following international commercial seroconversion panels from BBI (USA) were tested::

Panel	Sample number	HBsAg ELISA*	Subtype
PHM917	3	1 (43)	indet.
PHM920	6	4 (26)	*ad*
PHM927	6	4 (7)	indet.
PHM928	7	3 (14)	*ad*
PHM930	5	4 (3)	*ad*
PHM933M	5	4 (7)	*ad*
PHM934	6	5 (3)	*ad*

*The number of reactive samples by ELISA is indicated. The number between brackets indicates the number of days from the initial bleeding and the first reactive sample.

Analytical sensitivity (lower detection limit)

It has been calculated with the following international preparations
- Second International Standard for HBsAg, subtype adw2,

genotype A. NIBSC code 00/588:
Diluted in human serum negative for HBsAg and anti-HBs detected up to 0.10 IU/ml.
- Standards for HBsAg subtypes ad and ay provided by Paul Erlich Institute (from preparations of 100 U/ml and 50,000 U/ml, respectively):
Diluted in bovine serum detected up to 0.063 U/ml dilution of ad subtype and up to 0.12 U/ml dilution of ay subtype.
- Hepatitis B Surface Antigen Sensitivity Panel PHA808, BBI, USA:
Samples 1-8 were detected (up to 0.06 IU/ml ad subtype), and 11-17 (up to 0.09 IU/ml subtype ay).

Specificity

In a study performed on 685 sera and plasma samples from outpatients, the obtained specificity was 100% (IC95%: 99.93-100).
In a study performed on 1134 sera samples from blood banks, the obtained specificity was 99.82% (IC95%: 99.54-100).
In a further study on 1256 plasma samples from two different health centers, a specificity of 99.68% was obtained (IC95%: 99.33-100).

A possible cross-reactivity was evaluated, assaying samples from 364 individuals with different clinical conditions that may be the cause of unspecific reactions for HBsAg ELISA. This group included samples:
- With antibodies to HCV, EBV, CMV, HIV, HTLV and other viruses.
- With different autoantibodies (AGA, AMA, ATA, ACA, TPO, and others).
- With antibodies to *Treponema pallidum*, *Mycoplasma pneumoniae*, *Trypanosoma cruzi*, and other microorganisms.
- From hemodialyzed patients and pregnant women.

The obtained specificity for this population was 99.73% (IC95%: 99.05-100).

Precision

The test precision was evaluated following EP5-A protocol recommended by the CLSI (former NCCLS). The assays were performed with samples having different HBsAg ELISA reactivity levels and with controls. Two daily assays were performed testing each sample by duplicate during 20 days

	Mean absorb.	Intra-assay		Total	
		S.D.	C.V.	S.D.	C.V.
Sample 1	0,277	0,012	4,44%	0,032	11,44%
Sample 2	0,930	0,031	3,32%	0,113	12,11%
Sample 3	1,171	0,050	4,30%	0,117	10,02%
Control (+)	1,880	0,068	3,62%	0,146	7,79%
Control (-)	0,026	0,002	8,53%	0,004	16,83%

SD: standard deviation, CV: coefficient of variation
n= 80

WIENER LAB. PROVIDES

Kit for 96 tests (Cat. Nº 1483254).
Kit for 192 tests (Cat. Nº 1483260).

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SYMBOLS EXPLANATION

Policubeta	Sensib.

Coated microtitration plate

TMB	Diluy.

TMB Diluent

Conjugado	Conc.

Concentrated Conjugate

Conjugado	Diluy.

Conjugate Diluent

TMB

TMB

Buf. Lavado	Conc.

Concentrated Wash Buffer

Control	+

Positive Control

Control	-

Negative Control

Stopper

Stopper

The following symbols are used in the packaging for Wiener lab. diagnostic reagents kits.

CE This product fulfills the requirements of the European Directive 98/79 EC for "in vitro" diagnostic medical devices

EC REP Authorized representative in the European Community

IVD "In vitro" diagnostic medical device

Contains sufficient for <n> tests

Use by

Temperature limitation (store at)

Do not freeze

Biological risks

Volume after reconstitution

Cont. Contents

LOT Batch code

Manufactured by:

Harmful

Corrosive / Caustic

Irritant

Consult instructions for use

Calibr. Calibrator

CONTROL Control

CONTROL + Positive Control

CONTROL − Negative Control

REF Catalog number

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