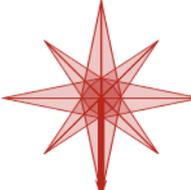


bi-x-act [®] OF FINLAND	HBsAg ELISA 96 & 480 well plate Kit (TMB)
CE IVD	Productcodes: 2HBsAg96 2HBsAg480
E  Web	info@ekoweb.fi phone +358 50 5645670 fax +358 2 2433786

Instruction for use

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1) Intended Use

HbsAg ELISA 96-well plate kit (TMB) is an enzyme immunoassay diagnostic kit for in vitro qualitative detection of hepatitis B surface antigen (HbsAg) in human serum or plasma (heparin, citrate or EDTA).

2) Summary and test explanation

The hepatitis B surface antigen (HBsAg) is the first marker that appears in the blood following infection with hepatitis B virus (HBV) some days or weeks before clinical symptoms manifest. It is a lipoprotein polypeptide which constitutes the external envelope of the HB virus. The detection of HBsAg in human serum or plasma indicates an ongoing HBV infection, either acute or chronic. Testing of additional HBV markers is needed to define the specific disease state. HBsAg assays are used not only to diagnose HBV infections but also to monitor the course of the disease and the efficacy of antiviral therapy.

HbsAg ELISA (TMB) kit is a fast test for the qualitative detection of the presence of HBsAg in serum or plasma (heparin, citrate or EDTA) specimen. The test utilizes monoclonal and polyclonal (anti-guinea pig) antibodies to selectively detect elevated levels of HBsAg in serum or plasma.

Specimens which are non-reactive by HBsAg ELISA test are considered negative for HBsAg. Specimens with positive reaction should be retested in duplicate.

In case of a reactive repeat reaction, the specimen should be confirmed for HBsAg reactivity with validated confirmatory reagents.

Only confirmed positive specimens are considered to contain HBsAg.

3) Test description

HBsAg ELISA (TMB) is a solid-phase enzyme immunoassay (ELISA= enzyme-linked immunosorbent assay) based on the sandwich principle.

The solid phase of the microtiter plate is made of polystyrene wells coated with mouse monoclonal antibodies specific for HBsAg; whereas guinea pig polyclonal antibody purified by affinity chromatography is used to prepare the anti-HBs-peroxidase (horseradish) conjugate in the liquid-phase.

When a serum or plasma specimen containing HBsAg is added to the anti-HBs antibody-coated wells together with the peroxidase conjugated anti-HBs antibody and incubated, an antibody-HBsAg-antibody-peroxidase complex will form on the wells.

After washing the microtiter plate to remove unbound material, a solution of TMB substrate is added to the wells and incubated. A colour develops in proportion to the amount of HBsAg bound to Anti-HBs. The peroxidase-TMB reaction is stopped by addition of sulphuric acid. The optical density of developed colour is read with a suitable photometer at 450nm with a selected reference wavelength within 620 to 690nm.

The test principle is shown also in the following figure:

A) Specimen containing HBsAg:

1. Plate well (Anti-HBs) + specimen (HBsAg) + Anti-HBs-peroxidase → Anti-HBs-HBsAg (Anti-HBs-peroxidase) sandwich complex

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2. Sandwich complex + TMB substrate solution → Light blue or blue colour
3. Add sulphuric acid to stop the colour development → Read OD at 450nm (reference wavelength 620-690nm)

B) Specimen without HBsAg

1. Plate well (Anti-HBs) + specimen (no HBsAg) + Anti-HBs-peroxidase → Anti-HBs (on the well)
2. Anti-HBs (on the well) + TMB substrate solution → Colourless to light blue colour
3. Add sulfuric acid to stop the colour development → Read OD at 450nm (reference wavelength 620-690nm)

4) Description of Materials Provided

Items	Components	Description	Qt. per 96 tests
(1H)	Anti-HBs Plate	Microtiter plate coated with mouse monoclonal anti-HBs.	1 plate
(2H)	Anti-HBs – Peroxidase Solution	Polyclonal anti-HBsHRPO conjugate, diluted in buffer with protein stabilizers. Preservatives: 0.003% Gentamycin and 0.01% Thimerosal Dye: phenol red.	1 bottle, 6 ml
(3H)	CONTROL + HBsAg Positive Control	Inactivated human serum positive for HBsAg but non reactive for anti-HCV and anti-HIV1/2, diluted in buffer with protein stabilizers. Preservatives: 0.003% Gentamycin and 0.01% Thimerosal	1 bottle, 1.0 ml
(4H)	CONTROL - HB Negative Control	Serum non-reactive for HBV markers, anti-HCV and anti-HIV1/2, diluted in buffer with protein stabilizers. Preservatives: 0.003% Gentamycin and 0.01% Thimerosal	1 bottle, 1.5 ml
(7E)	2N H ₂ SO ₄	2N sulphuric acid	1 bottle, 10 ml
(10E)	Conc. Washing Solution D (20X)	Concentrated Phosphate buffer with Tween-20	1 bottle, 50 ml
(11E)	TMB Substrate Solution A	0.6mg/ml of 3,3',5,5'-tetramethylbenzidine (TMB) in an organic base.	1 bottle, 10 ml
(12E)	TMB Substrate Solution B	Citrate Acid Buffer containing 0.03% H ₂ O ₂	1 bottle, 10 ml

Accessories (provided as needed):

- Adhesive slips
- Absorbent pads
- Black cover

Other required materials, but not provided:

50 ul, 100 ul micropipettes and tips are needed

Water bath or incubator with temperature control at +37°C

Plate washing equipment

ELISA Microwell Reader:

Dual wavelength 450nm with 620-690nm as reference wavelength, bandwidth 10nm

Fully automatic EIA micro-plate analyzer is optional. User should validate the automatic EIA micro-plate analyzer in combination with the kit.

Storage condition and stability of the kit and components:

Kit/components	Storage temp.	State	Stability
HBsAg ELISA -96 (TMB) Kit	+2 - +8° C	Original Once open	15 months 1 month
HBsAg Positive Control	+2 - +8° C	Original Once open	15 months 1 month
HB negative Control	+2 - +8° C	Original Once open	15 months 1 month
Anti-HBs Plate	+2 - +8° C	Original Once open	24 months 1 month
Anti-Hbs-HRPO Conjugate Solution	+2 - +8° C	Original Once open	15 months 1 month
Concentrated Washing Solution D (20X)	Room temp.	Original Once open	24 months 1 month
20 x Diluted Washing solution	Room. temp. +2 - +8° C	Diluted Diluted	2 days 1 week
TMB substrate Solution A	+2 - +8° C	Original Once open	18 months 1 month
TMB substrate Solution B	+2 - +8° C	Original Once open	18 months 1 month
2N Sulfuric Acid	Room temp.	Original Once open	24 months 1 month

5) Instructions for use

5.1) Warnings:

5.1.1) This kit is for professional use only

5.1.2) This kit is for in vitro diagnosis only

5.1.3) Bring all kit reagents and samples to room temperature (+20 to +30°C) and mix carefully before use.

5.1.4) Do not use reagent beyond its expiration date.

5.1.5) Do not interchange reagents between different lots.

5.1.6) Do not put pipette in mouth.

5.1.7) Do not smoke or eat in areas where specimens or reagents are handled.

5.1.8) All kit components and specimens should be regarded as potential health hazards. It should be used and discarded according to your laboratory's safety procedures. Such safety procedures probably include the wearing of protective gloves and avoiding the use of aerosols.

5.1.9) Potential infectious specimens and non-acid containing spills or leakages should be wiped up thoroughly with 5% sodium hypochlorite or treated in accordance with your practice for potential bio-hazard control.

5.1.10) Prior to disposing used specimens and kit reagents as general waste; it should be treated in accordance with the local practice of potential bio-hazardous waste or treated as follows:

Both liquid and solid waste should be autoclaved at +121° C for at least 30 minutes.

Solid waste can also be incinerated.

Non-acidic liquid waste can be treated with sodium hypochlorite diluted to a final concentration of 1 %.

Acidic liquid wastes must be neutralized before treatment with sodium hypochlorite as mentioned above and should stand for 30 minutes to obtain effective disinfection.

5.1.11) 2N Sulfuric Acid is an irritant to skin, eyes, respiratory tract and mucous membranes.

Avoid contact of the 2N sulfuric acid with skin and mucous membranes. In case of contact, flush immediately with abundant amounts of water. In case of inhalation, find fresh air and seek medical attention in case of pain.

5.1.12) TMB substrate solution A contains organic solvent, which is flammable. TMB substrate solution A contains dimethyl sulfoxide, an irritant to skin and mucous membranes.

5.1.13) Although all human sourced material are tests non reactive for Anti-HCV and Anti-HIV, and inactivated at +56° C for one hour, the reagent shall be handled as potential infectious material.

5.2) Specimen collection and preparation for analysis

5.2.1) No special preparation of the patient is required prior to blood collection. Blood should be collected by approval medical techniques.

5.2.2) Either serum or plasma specimens can be used with this test kit. Whole blood specimen should be separated as soon as possible in order to avoid hemolysis. Any particulates (e.g. fibrin clots, erythrocytes) contained in the specimen should be removed prior to use.

5.2.3) Specimens must be stored at +2 to +8° C and avoid heat-inactivation to minimize deterioration. For long-term storage, they must be frozen below -20° C. Storage in self-defrosting freezer is not recommended.

5.2.4) Frozen specimens must be thoroughly thawed and mixed homogenously before test.

5.2.5) Avoid multiple freeze-thaw procedures.

Warning:

1. Specimen must not contain any compounds of AZIDE, which inhibits the peroxidase activity.
2. Incompletely coagulated sera and microbial-contaminated specimens should not be used.

5.3) Storage conditions and stability of reagents

5.3.1) The kit must be stored at +2 to +8° C. Do not freeze.

5.3.2) Strips of the plate should be used within one month after opening the original aluminium foil bag. The unused strips should be kept in the aluminium foil bag and taped tightly.

5.3.3) Return reagents to +2 to +8° C immediately after use.

5.3.4) Washing Solution D (20x) Concentrate can be stored at room temperature to avoid crystallization, because the kits are stored at +2 to +8° C. If crystals have been precipitated before use, warm up the solution in 37° C water bath till the crystals dissolve.

5.4) Plate washing procedure

5.4.1) Preparation of washing solution:

Dilute Washing Solution D (20X) Concentrate with distilled or de-ionized water to obtain a 1:20 dilution. Do not use tap water.

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5.4.2) Plate washing:

(a) For plate washer with overflow aspirating function: 6 cycles with at least 0.5 ml washing buffer per well per cycle.

or

(b) For plate washer without overflow aspirating function: 8 cycles with at least 0.35 ml washing buffer per well per cycle.

5.4.3) Blot dry by inverting the plate and tapping firmly onto absorbent paper. Too much residual wash buffer will cause false results.

Warning:

Improper washing will cause false results.

5.5) Test procedure

5.5.1) Bring all reagents and specimens at room temperature (+20 to +30° C) before assay. Adjust water bath or incubator to +37 +/- 1 ° C.

5.5.2) Reserve one well for blank. Add 50ul of each control or specimen to appropriate wells of the microtiter plate (3 negative controls and 2 positive controls).

Note:

- a. Use a clean pipette tip for each sampling to avoid cross-contamination.
- b. Each plate needs respective negative controls, positive controls and blank well.
- c. Do not use any cut-off value established for other plates of HBsAg ELISA kits.

5.5.3) Add 50 ul of Anti-HBs-Peroxidase Solution to each well except the blank.

Note:

Do not touch the wall of the plate wells to prevent contamination.

5.5.4) Gently tap the plate.

5.5.5) Remove the protective backing from the Adhesive Slip and press it onto the reaction plate, so that it is tightly sealed.

5.5.6) Incubate the reaction plate in +37 (+/- 1)°C water bath or incubator for 80 minutes.

5.5.7) At the end of the incubation period, remove and discard the Adhesive Slip and wash the plate in accordance with 5.4) Plate washing procedure.

5.5.8) Select one of the following two method for colour development:

A. Mix equal volumes of TMB Substrate Solution A and B in a clean container immediately prior to use.

Add 100ul of the mixture solution to each well including the blank well.

B. Add 50 ul of TMB Substrate Solution A first, and then add 50 ul of TMB Substrate solution B into each well including the blank. Mix well carefully.

Note:

TMB Substrate Solution A should be colourless to light blue; otherwise; it should be discarded.

The mixture of TMB Substrate Solution A and B should be used within 30 minutes after mix. The mixture should be kept away from intense light.

5.5.9) Cover the plate with a black cover and incubate at room temperature for 30 minutes.

5.5.10) Stop the reaction by adding 100 ul of 2N H₂SO₄ to each well including the blank.

5.5.11) Determine the absorbance of controls and test specimens within 30 minutes with a precision spectrophotometer at 450/620-690nm (450nm reading wavelength with 620-690nm reference wavelength).

Use the blank well to blank spectrophotometer.

Note:

The colour of the blank should be colourless to light yellow; otherwise, the results are invalid.

(The reference wavelength of the photometer to be used can be 620 to 690 nm. However, the user should validate the photometer in combination with HBsAg ELISA (TMB) kit before use).

5.6) Calculations of results

5.6.1) Calculation of the NC (Mean Absorbance of Negative Control).

Example: Sample no.	Absorbance
1	0.022
2	0.025
3	0.023

$$NC = (0.022 + 0.025 + 0.023) / 3 = 0.023$$

NC should be ≤ 0.1 , otherwise the test is invalid!

5.6.2) Calculation of the PC (Mean Absorbance of Positive Control)

Example: Sample no.	Absorbance
1	1.432
2	1.508

$$PC = (1.432 + 1.508) / 2 = 1.470$$

PC should be ≥ 0.6 , otherwise, the test is invalid.

5.6.3) Calculation of the P – N value

$$P-N = PC - NC$$

Example: $NC = 0.024, PC = 1.470$

$$P-N = 1.470 - 0.024 = 1.446$$

P-N value must be ≥ 0.5 , otherwise, the test is invalid.

5.6.4) Calculation of the Cut off Value:

$$\text{Cut off Value} = NC + 0.025$$

Example: $\text{Cut off Value} = 0.023 + 0.025 = 0.048$

5.7) Validity of test runs

5.7.1) NC should be ≤ 0.1 ; otherwise, the test is invalid

5.7.2) PC should be ≥ 0.6 ; otherwise, the test is invalid

5.7.3) P – N Value must be ≥ 0.5 ; otherwise, the test is invalid

5.8) Interpretation of results

5.8.1) Specimens with absorbance values LESS than Cut off Value are NON-REACTIVE and are considered NEGATIVE for HBsAg.

5.8.2) Specimens with absorbance value GREATER than or EQUAL to the Cut off Value are considered INITIALLY REACTIVE. The original specimens must be retested in duplicate.

5.8.3) If both absorbance values in the retest are LESS than the Cut off Value, the specimens are considered NEGATIVE for HBsAg.

If in the retest at least one of the two absorbance values is GREATER than or EQUAL to the Cut off Value then the specimens are considered as repeated HBsAg positive. The repeated positive specimen shall be confirmed with certain valid confirmatory reagents.

5.9) Troubleshooting

If the result cannot be reproduced, perform a preliminary troubleshooting by checking the possibilities listed below:

- Improper washing procedure.
- Contamination with positive specimen.
- Wrong volume of sample, conjugate or substrates.
- Contamination of the well rim with conjugate.
- Improper specimen, such as hemolyzed serum or plasma, specimen containing sediments and specimen not thoroughly mixed before use.
- Wrong incubation time or temperature.
- Obstructed or partial obstructed washer aspirate/dispense head and needles.
- Insufficient aspiration.

5.10) Limitations and interferences

- 5.10.1) This reagent kit is to be used for un-pooled human serum or plasma only.
- 5.10.2) The reagent kit has not been validated for use with cadaveric samples.
- 5.10.3) A negative HBsAg result without other evidence should not be used to exclude an HBV infection.
- 5.10.4) Interfering substances:

The following results were obtained in respective experiments:

1. No interferences with different anticoagulants such as lithium heparin, EDTA, citrate have been observed.
2. Heat-treated specimens (+60° C, 10 hours) exhibited diminished HBsAg titer.
3. No cross reactivity was detected using specimens deriving from patients with a) other infections by HAV, EBV, CMV, HSV, VZV, Lyme Borreliosis, HCV, HIV b) other disease states as chronic renal failure, hemodialysis, autoimmune hepatitis, liver cirrhosis, and c) presence of certain antibodies like HAMA; GAD, IA2,APS.
4. samples containing potential interfering substances (e.g. triglycerides (lipemia), haemoglobin (hemolysis), bilirubin (icterus), monoclonal immunoglobulin components, elevated levels of autoimmune antibodies (rheumatoid factor-RF, antinuclear antibodies-ANA, or antimicrochondrial antibodies –ANA) and samples from pregnant women did not interfere with HBsAg ELISA (TMB) kit.

5.11) Performance characteristics

5.11.1) Diagnostic specificity

Results from the European performance evaluation for HBsAg ELISA (TMB) kit – reactivity of HBV negative donor and clinical specimens.

Total no. of specimens	N	Neg	IR	RR	Confirmed	False positive
HBV negative (clinical specimen)	213	211	2	2	0	2
HBV negative (donor specimen)	5501	5479	22	22	0	22
Total	5714	5690	24	24	0	24

IR: initial reactive, RR = repeat reactive

Diagnostic specificity = 5690/5714 = **99.58%**

5.11.2) Diagnostic sensitivity

The diagnostic sensitivity determined in the European performance evaluations yielded the following results:

Sample	No. of sample	Reactive	Sensitivity
HBsAg positive sera	400	400	100%

5.11.3) Analytical sensitivity determined using the PEI HBsAg standard 0.05 PEI U/ml= 0.2ng/ml

5.11.4) Precision

Intra-assay precision was determined using one positive control sample and two patient serum samples of different HBsAg concentration (slightly above the cut off level and at medium level) which were analyzed in replicates of 20 in a single run over 3 days.

The calculated CV's ranged between 3.7% and 11.36% (= acceptable value for an immunoassay in microtiter plate format).

Inter-assay precision evaluation experiments were performed over 10 operating days in using five serum samples (with borderline positive and clearly above cut off value HBsAg levels)

The calculated CV's ranges between 30.4 for an HBsAg negative and 23.1% for HBsAg low positive sample (= acceptable values for inter-assay imprecision of an immunoassay in microtiter plate format).

5.11.5) Antigen Excess/High –dose hook effect

This was performed testing a serum sample with a very high HBsAg concentration of 125 mg/l in serial dilution with the HBsAg ELISA (TMB) assay. No high-dose hook effect was observed.

5.12) Flow chart of the test procedure

