

bi-x-act[®]
of FINLAND

Anti-HBs ELISA 96 and
480 Well Plate Test Kit



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1) Intended Use

Anti-HBs ELISA Test is an enzyme immunoassay diagnostic kit for in vitro qualitative detection of Antibody to Hepatitis B surface antigen (Anti-HBs) in human serum or plasma (heparin, citrate or EDTA).

The test can be used prior and after hepatitis B vaccination to assess the immune status as well as an aid to diagnose and monitor infections by hepatitis B virus.

2) Summary and Test Explanation

Hepatitis B surface antigen (HBsAg) is the first antigen to appear following infection by hepatitis B virus. The development of antibodies against HBsAg (anti-HBs) occurs in 90% of patients infected with HBV late in convalescence approx. 3 to 4 months after the onset of the disease and is associated with resolution of the infection and protective immunity. The absence of anti-HBs is indicative of susceptibility to HBV infection, and can identify individuals who may benefit from vaccination. WHO reports that people with an anti-HBs titer of 10 mIU/mL can be assumed to be protected against HBV infection. The measurement of anti-HBs is useful for pre-immunization screening as well as to establish seroconversion after an infection or following active immunization with hepatitis B vaccines. Anti-HBs titers of < 100 mIU/mL will identify inadequate responders who require booster vaccination within one year. In addition, anti-HBs testing is useful to monitor the course of disease following acute HBV infection.

Anti-HBs ELISA Test is a fast test for the qualitative detection of the presence of antibodies to HBsAg in serum or plasma (heparin, citrate or EDTA) specimens. The test utilizes HBsAg on the wells and as peroxidase-conjugate. Specimens which are non-reactive by **Anti-HBs ELISA Test** are considered negative for Anti-HBs. Specimens with positive reaction should be retested in duplicate.

The test has to be repeated in duplicate for specimens with absorbance value within the Retest Range (Cutoff Value $\pm 10\%$).

3) Test Description

Anti-HBs ELISA Test is a solid-phase enzyme immunoassay (ELISA= enzyme-linked immuno-sorbent assay) based on the "sandwich principle". The solid phase of the microtiter plate is made of polystyrene wells coated with HBsAg (subtype Ad and Ay), and the liquid phase of peroxidase conjugated HBsAg (subtype Ad and Ay).

When a serum or plasma specimen containing Anti-HBs is added to the HBsAg-coated wells together with the peroxidase conjugated HBsAg and incubated, (antigen)-(Anti-HBs)-(antigen • peroxidase) complexes 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 color develops in proportion to the amount of Anti-HBs bound to HBsAg. The peroxidase-TMB reaction is stopped by addition of sulfuric acid. The optical density of developed color is read with a suitable photometer at 450 nm with a selected reference wavelength within 620 to 690 nm^{*1}.

The above test principle is shown also in the following figure.

A Specimen containing Anti-HBs:

1. Plate well (HBsAg) + specimen (Anti-HBs) + HBsAg • peroxidase
→ HBsAg • Anti-HBs • (HBsAg • peroxidase) sandwich complex
2. Sandwich complex + TMB substrate solution
→ Light blue to blue color
3. Add sulfuric acid to stop the color development
→ Read OD at 450 nm/620-690 nm^{*7}

B Specimen without Anti-HBs:

1. Plate well (HBsAg) + specimen (no Anti-HBs) + HBsAg • peroxidase
→ HBsAg (on the well)
2. HBsAg (on the well) + TMB substrate solution
→ Colorless to light blue color
3. Add sulfuric acid to stop the color development
→ Read OD at 450 nm/620-690 nm^{*7}

4) Description of Provided Materials & Product Code System

Item 1 - 6 on the following reagent table should be refrigerated at +2 to +8°C . Washing Solution D (20x) and 2N H₂SO₄ can be stored at +2 to +30°C.

ITEMS	Components	Description	Qt. per 96 tests
(1)	HBsAg Plate	Microtiter plate coated with HBsAg.	1 plate
(2)	HBsAg • Peroxidase Solution	HBsAg • HRPO conjugate, diluted in buffer with protein stabilizers. Preservatives: 0.003% Gentamycin 0.01% Thimerosal.	1 bottle, 7 ml

(3)	Anti-HBs Positive Control	Inactivated human Anti-HBs positive serum diluted in buffer with protein stabilizers. Preservatives: 0.003% Gentamycin and 0.01% Thimerosal.	1 bottle, 1.1 ml
(4)	HB Negative Control	Serum non-reactive for HBV markers in buffer with protein stabilizers. Preservatives: 0.003% Gentamycin and 0.01% Thimerosal.	1 bottle, 1.6 ml
(5)	TMB Substrate Solution A	0.6 mg/ml of 3, 3', 5, 5'-tetramethylbenzidine (TMB) in an organic base.	1 bottle, 10 ml
(6)	TMB Substrate Solution B	Citrate Acid Buffer containing 0.03% H ₂ O ₂ .	1 bottle, 10 ml
(7)	Conc. Washing Solution D (20x)	Concentrated Phosphate buffer with Tween-20	1 bottle 52 ml
(8)	2N H ₂ SO ₄	2N H ₂ SO ₄ (Sulfuric Acid)	1 bottle 12 ml

ACCESSORIES: (provided as needed)

ITEMS	Components
(9)	Adhesive slips
(10)	Absorbent pads
(11)	Black cover

OTHER MATERIALS REQUIRED, BUT NOT PROVIDED

ITEMS	Components
(1)	50 µl, 100 µl micropipettes and tips are needed.
(2)	Waterbath or incubator with temperature control at +37°C.
(3)	Plate washing equipment.
(4)	ELISA microwell reader: Dual wavelength 450nm with 620-690nm as reference wavelength, bandwidth 10nm ^{*1} .
(5)	Fully automatic EIA micro-plate analyzer is optional. User should validate the automatic EIA micro-plate analyzer in combination with the kit.

4.1) Storage Condition and Stability of the kit

Kit/components	Storage condition	State	Stability
Anti-HBs ELISA Test Kit	+2 to +8°C	Original	15 months
		Once open	1 month
Anti-HBs Positive Control	+2 to +8°C	Original	15 months
		Once open	1 month
HB Negative Control	+2 to +8°C	Original	15 months
		Once open	1 month
HBsAg Plate	+2 to +8°C	Original	24 months
		Once open	2 month
HBsAg • HRPO Conjugate Solution	+2 to +8°C	Original	15 months
		Once open	1 month
Concentrated Washing Solution D (20X)	Room temp.	Original	24 months
		Once open	1 month
20X Diluted Washing Solution	Room temp.	Diluted	2 days
	+2 to +8°C	Diluted	1 week
TMB Substrate Solution A	+2 to +8°C	Original	18 months
		Once open	1 month
TMB Substrate solution B	+2 to +8°C	Original	18 months

		Once open	1 month
2N Sulfuric Acid	Room temp.	Original	24 months
		Once open	1 month

5) Instructions for Use

5.1) Warning:

- 5.1.1) This reagent kit is for professional use only.
- 5.1.2) This reagent kit is for *in vitro* diagnostic use 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 the pipette in your mouth.
- 5.1.7) Do not smoke or eat in areas where specimens or reagents are handled.
- 5.1.8) The positive control, negative control, conjugate solution and specimens should be regarded as potential health hazards. It should be used and discarded according to your own laboratory's safety procedures. Such safety procedures may include the wearing of protective gloves and avoiding the generation of aerosols.
- 5.1.9) Potential infectious specimens and nonacid containing spills or leakages should be wiped up thoroughly with 5% sodium hypochlorite or treated in accordance with the local procedures 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 procedures for potential bio-hazardous waste or treated as follows:
Both liquid and solid waste should be autoclaved maintaining +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, clean with large lots of water immediately. In case of inhalation, supply fresh air and seek medical advice in case of complaints.
- 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 material of human source is tested nonreactive for Anti-HCV and Anti-HIV, and inactivated at +56°C for one hour, the reagent shall be handled as potential infectious material.*²

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 approved 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, specimens should be frozen below -20°C. Storage in self-defrosting freezer is not recommended.
- 5.2.4) Frozen specimens must be thoroughly thawed and mixed homogeneously before testing.
- 5.2.5) Avoid multiple freeze-thaw procedures
- 5.2.6) **WARNING**
 - 1. The specimen must not contain any AZIDE compounds, which can inhibit the peroxidase activity of the conjugate.
 - 2. Incompletely coagulated serum samples and microbial-contaminated specimens should not be used.

5.3) Reagents Storage

- 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 2 months after opening the original aluminum foil bag. The unused strips should be kept in the aluminum 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 and shipped 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 1:20 dilution. Do not use tap water.
- 5.4.2) Plate washing:
 - (a) For plate washer with overflow aspirating function: 6 cycles with at least 0.5ml 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 in the wells will cause false results.

WARNING

Improper washing will cause false results.

5.5) Test procedure

- 5.5.1) Bring all reagents and specimens to 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 50µl of each control or specimen to appropriate wells of reaction 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 its own negative controls, positive controls and blank well.
 - c) Do not use any cut-off value established for other plates of **Anti-HBs ELISA Test**
- 5.5.3) Add 50 µl of HBsAg • Peroxidase solution to each well except the blank.

NOTE:

Do not touch the well wall for preventing 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 a +37±1°C water bath or incubator for 1 hour.
- 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 methods for color development:
 - A. Mix equal volumes of **TMB Substrate Solution A and B** in a clean container immediately prior to use. Add **100 µl** of the mixture solution to each well including the blank well.
 - B. Add **50 µl** of **TMB Substrate Solution A** first, and then add **50 µl** of **TMB Substrate Solution B** into each well including the blank. Carefully mix well.

NOTE:

TMB Substrate Solution A should be colorless 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 avoided 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 100µl 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 photometer at 450 / 620-690 nm (450 nm reading wavelength with 620-690 nm reference wavelength)*7. Use the blank well to blank photometer.

NOTE: The color of the blank should be colorless to light yellowish; otherwise, the test results are invalid.

Substrate blank : absorbance value must be less than 0.100.

5.6) Calculation of Test Results

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

Example: Sample No.	Absorbance
1	0.015
2	0.016
3	0.014

$$NCx = (0.015 + 0.016 + 0.014) / 3 = 0.015$$

NCx must be ≤ 0.2 , otherwise, the test is invalid.

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

Example: Sample No.	Absorbance
1	0.846
2	0.902

$$PCx = (0.846 + 0.902) / 2 = 0.874$$

PCx must be ≥ 0.5 , otherwise, the test is invalid.

5.6.3) Calculation of the P - N Value

$$P - N = PCx - NCx$$

Example: $NCx = 0.015$

$PCx = 0.874$

$$P - N = 0.874 - 0.015 = 0.859$$

P - N Value must be ≥ 0.3 , otherwise, the test is invalid.

5.6.4) Calculation of the Cutoff Value

$$\text{Cutoff Value} = NCx + 0.025$$

Example: $\text{Cutoff Value} = 0.015 + 0.025 = 0.040$

5.6.5) Calculation of the Retest Range

$$\text{Retest Range} = \text{Cutoff Value} \pm 10\%$$

Example: $\text{Cutoff Value} = 0.040$

$$\text{Retest Range} = (0.040 - 0.004) \text{ to } (0.040 + 0.004) = 0.036 \text{ to } 0.044$$

5.7) Validity of the Test Runs

5.7.1) **NCx should be ≤ 0.2 ; otherwise, the test is invalid.**

5.7.2) **PCx should be ≥ 0.5 , otherwise, the test is invalid.**

5.7.3) **P - N Value must be ≥ 0.3 , otherwise, the test is invalid.**

NOTE: Negative Control: absorbance value must be less than or equal to 0.200 after subtracting the blank.

5.8) Interpretation of Results

5.8.1) Specimens with absorbance values less than (0.9 x Cutoff Value) are considered

NON-REACTIVE and are considered NEGATIVE for Anti-HBs.

5.8.2) Specimens with absorbance value greater than (1.1 x Cutoff Value) are considered REACTIVE and are considered POSITIVE for Anti-HBs.

5.8.3) Specimens with absorbance value within the Retest Range (Cutoff Value \pm 10%) shall be repeated in duplicate and interpreted as above.

Specimens with any of the repeat results in the retest range are reported as "indeterminate". It is suggested to test follow-up samples for "indeterminate" results.

5.9) Troubleshooting

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

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

5.10) Limitations and Interferences

- 5.10.1) This reagent kit is to be used for unpooled human serum or plasma only.
- 5.10.2) The reagent kit has not been validated for use with cadaveric samples.
- 5.10.3) Non-repeatable false positive results may be obtained with any enzyme immunoassay kit, largely due to technical error either from the part of the operator or malfunction of apparatus used.
- 5.10.4) Repeatable false reactive results ($\leq 2\%$) may occasionally be obtained.
- 5.10.5) An Anti-HBs negative result without other evidence does not preclude the possibility of previous infection with hepatitis B virus.
- 5.10.6) A (low) positive result in the **Anti-HBs ELISA Test** is no proof of protection and such it should be not used to exclude an infection by hepatitis B virus.
- 5.10.7) Anti-HBs positive specimens may not always show linear serial dilution properties as in serial dilution of standard material.

5.10.8) Potential interfering substances:

The following results were obtained in respective experiments:

1. No interferences with different anticoagulants such as lithium heparin, K-EDTA, sodium 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 a) with other infections by HAV, EBV, CMV, HSV, VZV, Lyme Borreliosis, HCV, HIV, b) with other disease states such as chronic renal failure, hemodialysis, autoimmune hepatitis, liver cirrhosis, and c) presenting certain antibodies like HAMA, GAD, IA2, APS).
4. Samples containing potential interfering substances [e.g. triglycerides (lipemia), hemoglobin (hemolysis), bilirubin (icterus), monoclonal immunoglobulin components, elevated levels of autoimmune antibodies (rheumatoid factor-RF, antinuclear antibodies-ANA, or antimito-chondrial antibodies-ANA)] and samples from pregnant women did not interfere with the **Anti-HBs ELISA Test**.

5.11) Performance characteristics

5.11.1) Diagnostic Specificity

Results from Evaluation for **Anti-HBs ELISA Test** AntiHBs - Reactivity of HBV Negative "Donor" and "Clinical" Specimens.

Anti-HBs negative Sample	No. of sample	Negative
Unselected donor samples	400	400
Hospitalized patients	134	134
Potential interfering samples	50	49
Total	584	583

$$\text{Diagnostic specificity} = 583/584 = 99.83\%$$

5.11.2) Analytical Sensitivity:

Detection limit determined using dilutions of Anti-HBs Standards.

The analytical sensitivity was determined to be 3.6 mIU/ml of anti-HBs for the **Anti-HBs ELISA Test** using among others the PEI Anti-HBs Standard.

The S/CO for 10 mIU/ml of anti-HBs was about 1.60. For routine testing, it may be useful and practical to increase the cutoff value for the positive result to 1.6x standard/CO in order to achieve a cutoff of approximately 10 mIU/ml of Anti-HBs (“protective titer”).

5.11.3) Test Linearity using blood samples

Linearity was evaluated using two high-titer Anti-HBs-positive serum samples by diluting them throughout the measuring range of the assay and then around the cutoff level in narrow dilution steps.

Anti-HBs ELISA Test showed linear behavior on dilution between 3.6 and 240 mIU/ml.

5.11.4) Diagnostic Sensitivity

Positive specimens/Specimens used to evaluate the sensitivity/ Patients with HBV infection

5.11.4.1) HBV infected individuals

Anti-HBs positive samples	No. of samples	Positive results
Natural infected individuals	125	125
Hep B vaccinated individuals	111	111
Total	235	235

Diagnostic sensitivity = 235/235= 100%

5.11.4.2) Commercial HBV seroconversion panels

4 commercially available HBV seroconversion panels consisting of follow-up samples which were collected at weekly or monthly intervals from patients suffering from acute hepatitis B, have been used. Anti-HBs ELISA Test was more sensitive than the Anti-HBs reference assay.

5.11.4.3) Follow-up samples

Samples were obtained from 22 healthy subjects who were vaccinated for hepatitis B. The testing was performed in samples from bleedings obtained before and 3 and 6 months after hepatitis B vaccination. A seroconversion for Anti-HBs was detected in all 22 subjects in the 3 months bleedings using Anti-HBs ELISA Test. On the other side, testing with the Anti-HBs reference assay showed seroconversion for 20 subjects in the 3 months after collection while for two further subjects the seroconversion was observed only in the samples obtained 6 months after vaccination. This indicates that Anti-HBs ELISA Test is more sensitive than the Anti-HBs reference assay.

5.11.5) Evaluation of Precision

5.11.5.1) Accuracy: intra-run repeatability and inter-run reproducibility

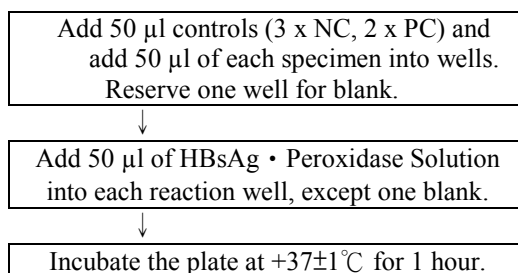
The positive control of the Anti-HBs ELISA Test (800 mIU/ml) and two serum samples with Anti-HBs levels just above cutoff and at medium level were tested in replicates of 20 in a single run over three days. The results were used to calculate the intra-run repeatability and inter-run reproducibility as presented in the following tables.

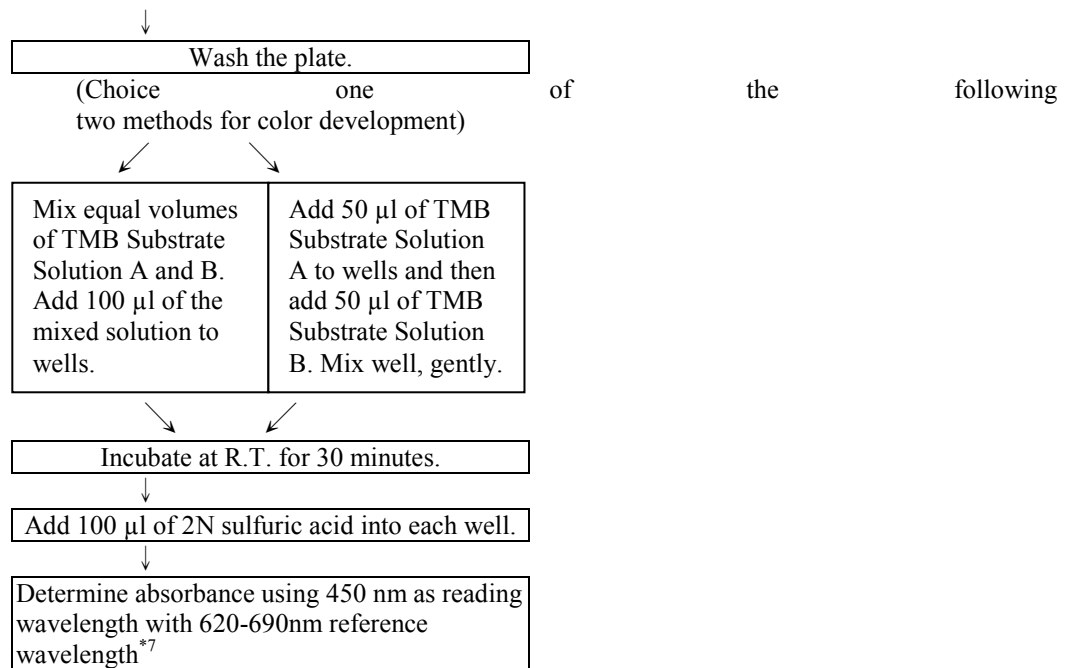
Item tested		Sample size	precision
Positive Control	intra-run	N = 20	CV ≤ 10.27%
	inter-run	N = 60	CV ≤ 7.11%
Patient Serum #1	intra-run	N = 20	CV ≤ 7.57%
	inter-run	N = 60	CV ≤ 7.17%
Patient Serum #2	intra-run	N = 20	CV ≤ 9.05%
	inter-run	N = 60	CV ≤ 7.71%

5.11.6) Traceability

The anti-HBs Calibrator has been calibrated against the Anti-HBs-IgG Standard using the Anti-HBs ELISA Test. The relative potency of the Anti-HBs Standard versus the anti-HBs Calibrator is 1.205 (1.117-1.297 95% CI). The Anti-HBs concentration of the Positive Control of Anti-HBs ELISA Test has been determined against the anti-HBs Calibrator and was established with 800 mIU/ml ±20%.

5.12) Flow Chart of the Test Procedure





6) Notes

- *1 The reference wavelength of the photometer to be used can be 620 nm to 690 nm. However, the user should validate the photometer in combination with Anti-HBs ELISA Test before use.
- *2 Incomplete inactivation of hepatitis B virus after heat treatment at +60°C for 10 hours, J. Infect. Dis. 138:242-244.

7) Bibliography

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6. European Consensus Group on Hepatitis B immunity: Are booster immunisations needed for lifelong hepatitis B immunity? Lancet. 2000;355:561-565.
7. The reference wavelength of spectrometer could be 620nm to 690nm. However, user should validate the spectrometer in combination with this kit before use.