

HBsAg_{one}

**Third generation Enzyme Immunoassay
for the determination of
Hepatitis B surface Antigen or HBsAg
in human serum and plasma**

- for "in vitro" diagnostic use only -



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HBsAg One

A. INTENDED USE

Third generation Enzyme Immunoassay (ELISA) for the one-step qualitative determination of Hepatitis B surface Antigen in human plasma and sera. The kit may be used for the screening of blood units and the follow-up of HBV-infected patients.

For "in vitro" diagnostic use only.

B. INTRODUCTION

The World Health Organization (WHO) defines Hepatitis B Virus infection as follows:

"Hepatitis B is one of the major diseases of mankind and is a serious global public health problem. Hepatitis means inflammation of the liver, and the most common cause is infection with one of 5 viruses, called hepatitis A,B,C,D, and E. All of these viruses can cause an acute disease with symptoms lasting several weeks including yellowing of the skin and eyes (jaundice); dark urine; extreme fatigue; nausea; vomiting and abdominal pain. It can take several months to a year to feel fit again. Hepatitis B virus can cause chronic infection in which the patient never gets rid of the virus and many years later develops cirrhosis of the liver or liver cancer.

HBV is the most serious type of viral hepatitis and the only type causing chronic hepatitis for which a vaccine is available. Hepatitis B virus is transmitted by contact with blood or body fluids of an infected person in the same way as human immunodeficiency virus (HIV), the virus that causes AIDS. However, HBV is 50 to 100 times more infectious than HIV. The main ways of getting infected with HBV are: (a) perinatal (from mother to baby at the birth); (b) child-to-child transmission; (c) unsafe injections and transfusions; (d) sexual contact.

Worldwide, most infections occur from infected mother to child, from child to child contact in household settings, and from reuse of un-sterilized needles and syringes. In many developing countries, almost all children become infected with the virus. In many industrialized countries (e.g. Western Europe and North America), the pattern of transmission is different. In these countries, mother-to-infant and child-to-child transmission accounted for up to one third of chronic infections before childhood hepatitis B vaccination programmes were implemented. However, the majority of infections in these countries are acquired during young adulthood by sexual activity, and injecting drug use. In addition, hepatitis B virus is the major infectious occupational hazard of health workers, and most health care workers have received hepatitis B vaccine.

Hepatitis B virus is not spread by contaminated food or water, and cannot be spread casually in the workplace. High rates of chronic HBV infection are also found in the southern parts of Eastern and Central Europe. In the Middle East and Indian sub-continent, about 5% are chronically infected. Infection is less common in Western Europe and North America, where less than 1% are chronically infected.

Young children who become infected with HBV are the most likely to develop chronic infection. About 90% of infants infected during the first year of life and 30% to 50% of children infected between 1 to 4 years of age develop chronic infection. The risk of death from HBV-related liver cancer or

cirrhosis is approximately 25% for persons who become chronically infected during childhood. Chronic hepatitis B in some patients is treated with drugs called *interferon* or *lamivudine*, which can help some patients. Patients with cirrhosis are sometimes given liver transplants, with varying success. It is preferable to prevent this disease with vaccine than to try and cure it.

Hepatitis B vaccine has an outstanding record of safety and effectiveness. Since 1982, over one billion doses of hepatitis B vaccine have been used worldwide. The vaccine is given as a series of three intramuscular doses. Studies have shown that the vaccine is 95% effective in preventing children and adults from developing chronic infection if they have not yet been infected. In many countries where 8% to 15% of children used to become chronically infected with HBV, the rate of chronic infection has been reduced to less than 1% in immunized groups of children. Since 1991, WHO has called for all countries to add hepatitis B vaccine into their national immunization programs."

Hepatitis B surface Antigen or HBsAg is the most important protein of the envelope of Hepatitis B Virus, responsible for acute and chronic viral hepatitis.

The surface antigen contains the determinant "a", common to all the known viral subtypes, immunologically distinguished by two distinct subgroups (ay and ad).

The ability to detect HBsAg with high sensitive immunoassays in the last years has led to an understanding of its distribution and epidemiology worldwide and to radically decrease the risk of infection in transfusion.

C. PRINCIPLE OF THE TEST

A mouse monoclonal antibody specific for HBsAg is fixed to the surface of microwells. Patient's serum/plasma is added to the microwell together with a second mouse monoclonal antibody, conjugated with Horseradish Peroxidase (HRP) and directed against a different epitope.

The specific immunocomplex, formed in the presence of HBsAg in the sample, is captured by the solid phase.

At the end of the one-step incubation, microwells are washed to remove unbound serum proteins and HRP conjugate.

The chromogen/substrate is then added and, in the presence of captured HBsAg immunocomplex, the colorless substrate is hydrolyzed by the bound HRP conjugate to a colored end-product. After blocking the enzymatic reaction, its optical density is measured by an ELISA reader.

The color intensity is proportional to the amount of HBsAg present in the sample.

D. COMPONENTS

The standard configuration of SAG1.CE contains reagents to perform 192 tests.

1. Microplate **MICROPLATE**

n° 2 12 strips of 8 breakable wells coated with anti HBsAg, affinity purified mouse monoclonal antibody, specific to "a" determinant, and sealed into a bag with desiccant.

2. Negative Control **CONTROL -**

1x4.0ml/vial Ready to use control. It contains goat serum, 10 mM phosphate buffer pH 7.4+/-0.1, 0.09% Na-azide and 0.1% Kathon GC as preservatives. The negative control is pale yellow color coded.

3. Positive Control **CONTROL +**

1x4.0ml/vial Ready to use control. It contains goat serum, non infectious recombinant HBsAg, 10 mM phosphate buffer pH 7.4+/-0.1, 0.02% gentamicine sulphate and 0.1% Kathon GC as preservatives. The positive control is color coded green.

4. Calibrator CAL ...

n°2 vials Lyophilized calibrator. To be dissolved with EIA grade water as reported in the label. Contains fetal bovine serum, non infectious recombinant HBsAg at 0.5 IU/ml (2nd WHO international standard for HBsAg, NIBSC code 00/588), 10 mM phosphate buffer pH 7.4+/-0.1, 0.02% gentamicine sulphate and 0.1% Kathon GC as preservatives.

Note: The volume necessary to dissolve the content of the vial may vary from lot to lot. Please use the right volume reported on the label .

5. Wash buffer concentrate WASHBUF 20X

2x60ml/bottle 20X concentrated solution. Once diluted, the wash solution contains 10 mM phosphate buffer pH 7.0+/-0.2, 0.05% Tween 20 and 0.1% Kathon GC.

6. Enzyme Conjugate Diluent CONJ DIL

1x30ml/vial Ready to use and red color coded reagent. It contains 10 mM Tris buffer pH 6.8+/-0.1, 1% normal mouse serum, 5% BSA, 0.1% Kathon GC and 0.02% gentamicine sulphate as preservatives. The solution is normally opalescent.

7. Enzyme Conjugate CONJ 20X

1x1.5ml/vial 20X concentrated reagent. It contains Horseradish Peroxidase (HRP) labeled mouse monoclonal antibody to HBsAg, determinant "a", 10 mM Tris buffer pH 6.8+/-0.1, 5% BSA, 0.1% Kathon GC and 0.02% gentamicine sulphate as preservatives.

8. Chromogen/Substrate SUBS TMB

3x16ml/vial. It contains a 50 mM citrate-phosphate buffered solution at pH 3.5-3.8, 4% dimethylsulphoxide, 0.03% tetra-methyl-benzidine (TMB) and 0.02% hydrogen peroxide (H₂O₂).

Note: To be stored protected from light as sensitive to strong illumination.

9. Sulphuric Acid H₂SO₄ 0.3 M

1x32ml/vial. It contains 0.3 M H₂SO₄ solution.
Attention: Irritant (Xi R36/38; S2/26/30)

10. Plate sealing foils: n°4

11. Package insert: n°1

Important note: Only upon specific request , Dia.Pro can supply reagents for 96, 480, 960 tests , as reported below:

Microplates	N°1	N°5	N°10
Negative Control	1x2ml/vial	1x10ml/vial	1x20ml/vial
Positive Control	1x2ml/vial	1x10ml/vial	1x20ml/vial
Calibrator	N° 1 vial	N° 5 vials	N° 10 vials
Wash buffer concentrate	1x60ml/vial	2x150ml/vial	4x150ml/vial
Enzyme conjugate	1x0.8ml/vial	1x4ml/vial	2x4ml/vial
Conjugate Diluent	1x16ml/vial	1x80ml/vial	2x80ml/vial
Chromogen/Substrate	1x25ml/vial	1x125ml/vial	2x125ml/vial
Sulphuric Acid	1x15ml/vial	1x80ml/vial	2x80ml/vial
Plate sealing foils	N° 2	N° 10	N° 20
Package insert	N° 1	N° 1	N° 1
Number of tests	96	480	960
Code	SAG1.CE.96	SAG1.CE.480	SAG1.CE.960

E. MATERIALS REQUIRED BUT NOT PROVIDED

1. Calibrated Micropipettes (150ul, 100ul and 50ul) and disposable plastic tips.
2. EIA grade water (double distilled or deionised, charcoal treated to remove oxidizing chemicals used as disinfectants).
3. Timer with 60 minute range or higher.
4. Absorbent paper tissues.

5. Calibrated ELISA microplate thermostatic incubator (dry or wet), capable to provide shaking at 1300 rpm+/-150, set at +37°C.
6. Calibrated ELISA microwell reader with 450nm (reading) and possibly with 620-630nm (blanking) filters.
7. Calibrated ELISA microplate washer.
8. Vortex or similar mixing tools.

F. WARNINGS AND PRECAUTIONS

1. The kit has to be used by skilled and properly trained technical personnel only, under the supervision of a medical doctor responsible of the laboratory.
2. When the kit is used for the screening of blood units and blood components, it has to be used in a laboratory certified and qualified by the national authority in that field (Ministry of Health or similar entity) to carry out this type of analysis.
3. All the personnel involved in performing the assay have to wear protective laboratory clothes, talc-free gloves and glasses. The use of any sharp (needles) or cutting (blades) devices should be avoided. All the personnel involved should be trained in biosafety procedures, as recommended by the Center for Disease Control, Atlanta, U.S. and reported in the National Institute of Health's publication: "Biosafety in Microbiological and Biomedical Laboratories", ed. 1984.
4. All the personnel involved in sample handling should be vaccinated for HBV and HAV, for which vaccines are available, safe and effective.
5. The laboratory environment should be controlled so as to avoid contaminants such as dust or air-born microbial agents, when opening kit vials and microplates and when performing the test. Protect the Chromogen (TMB) from strong light and avoid vibration of the bench surface where the test is undertaken.
6. Upon receipt, store the kit at 2.8°C into a temperature controlled refrigerator or cold room.
7. Do not interchange components between different lots of the kits. It is recommended that components between two kits of the same lot should not be interchanged.
8. Check that the reagents are clear and do not contain visible heavy particles or aggregates. If not, advise the laboratory supervisor to initiate the necessary procedures for kit replacement.
9. Avoid cross-contamination between serum/plasma samples by using disposable tips and changing them after each sample.
10. Avoid cross-contamination between kit reagents by using disposable tips and changing them between the use of each one.
11. Do not use the kit after the expiration date stated on the external container and internal (vials) labels. A study conducted on an opened kit has not pointed out any relevant loss of activity up to 6 re-use of the device and up to 6 months.
12. Treat all specimens as potentially infective. All human serum specimens should be handled at Biosafety Level 2, as recommended by the Center for Disease Control, Atlanta, U.S. in compliance with what reported in the Institutes of Health's publication: "Biosafety in Microbiological and Biomedical Laboratories", ed. 1984.
13. The use of disposable plastic-ware is recommended in the preparation of the liquid components or in transferring components into automated workstations, in order to avoid cross contamination.
14. Waste produced during the use of the kit has to be discarded in compliance with national directives and laws concerning laboratory waste of chemical and biological substances. In particular, liquid waste generated from the washing procedure, from residuals of controls and from samples has to be treated as potentially infective material and inactivated before waste. Suggested procedures of inactivation are treatment with a 10% final concentration of household bleach for 16-18 hrs or heat inactivation by autoclave at 121°C for 20 min..

15. Accidental spills from samples and operations have to be adsorbed with paper tissues soaked with household bleach and then with water. Tissues should then be discarded in proper containers designated for laboratory/hospital waste.

16. The Stop Solution is an irritant. In case of spills, wash the surface with plenty of water

17. Other waste materials generated from the use of the kit (example: tips used for samples and controls, used microplates) should be handled as potentially infective and disposed according to national directives and laws concerning laboratory wastes.

G. SPECIMEN: PREPARATION AND WARNINGS

1. Blood is drawn aseptically by venepuncture and plasma or serum is prepared using standard techniques of preparation of samples for clinical laboratory analysis. No influence has been observed in the preparation of the sample with citrate, EDTA and heparin.

2. Avoid any addition of preservatives to samples; especially sodium azide as this chemical would affect the enzymatic activity of the conjugate, generating false negative results.

3. Samples have to be clearly identified with codes or names in order to avoid misinterpretation of results. When the kit is used for the screening of blood units, bar code labeling and electronic reading is strongly recommended.

4. Haemolysed (red) and lipemic ("milky") samples have to be discarded as they could generate false results. Samples containing residues of fibrin or heavy particles or microbial filaments and bodies should be discarded as well as they could give rise to false positive results.

5. Sera and plasma can be stored at +2°.8°C for up to five days after collection. For longer storage periods, samples can be stored frozen at -20°C for several months. Any frozen sample should not be frozen/thawed more than once as this may generate particles that could affect the test result. If some turbidity is present or presence of microparticles is suspected after thawing, filter the sample on a disposable 0.2-0.8µ filter to clean it up for testing or use the two-steps alternative method.

H. PREPARATION OF COMPONENTS AND WARNINGS

A study conducted on an opened kit has not pointed out any relevant loss of activity up to 6 re-uses of the device and up to 6 months.

1. Microplates:

Allow the microplate to reach room temperature (about 1 hr) before opening the container. Check that the desiccant has not turned green, indicating a defect in conservation.

In this case, call Dia.Pro's customer service.

Unused strips have to be placed back inside the aluminum pouch, with the desiccant supplied, firmly zipped and stored at +2°.8°C. After first opening, remaining strips are stable until the humidity indicator inside the desiccant bag turns from yellow to green.

2. Negative Control:

Ready to use. Mix well on vortex before use.

3. Positive Control:

Ready to use. Mix well on vortex before use. The positive control does not contain any infective HBV as it is composed of recombinant synthetic HBsAg.

4. Calibrator:

Add the volume of ELISA grade water, reported on the label, to the lyophilized powder; let fully dissolve and then gently mix on vortex. The solution is not stable. Store the Calibrator frozen in aliquots at -20°C.

5. Wash buffer concentrate:

The 20x concentrated solution has to be diluted with EIA grade water up to 1200 ml and mixed gently end-over-end before use. As some salt crystals may be present into the vial, take care to dissolve all the content when preparing the solution.

In the preparation avoid foaming as the presence of bubbles could give origin to a bad washing efficiency.

Note: Once diluted, the wash solution is stable for 1 week at +2..8°C.

6. Enzyme conjugate:

The working solution is prepared by diluting 20X the concentrated reagent with the Enzyme Conjugate Diluent (e.g. 100 µl conjugate + 1.9 ml diluent).

Mix well on vortex before use.

The working solution is not stable. Avoid contamination of the liquid with oxidizing chemicals, dust or microbes. If this component has to be transferred, use only plastic, and if possible, sterile disposable containers.

Important note: Prepare only the volume necessary for the work of the day as the diluted tracer is not stable.

7. Chromogen/Substrate:

Ready to use. Mix well on vortex before use.

Avoid contamination of the liquid with oxidizing chemicals, air-driven dust or microbes. Do not expose to strong light, oxidizing agents and metallic surfaces.

If this component has to be transferred use only plastic, and if possible, sterile disposable container.

8. Sulphuric Acid:

Ready to use. Mix well on vortex before use.

Attention: Irritant (Xi R36/38; S2/26/30)

Legenda: R 36/38 = Irritating to eyes and skin.

S 2/26/30 = In case of contact with eyes, rinse immediately with plenty of water and seek medical advice.

I. INSTRUMENTS AND TOOLS USED IN COMBINATION WITH THE KIT

1. **Micropipettes** have to be calibrated to deliver the correct volume required by the assay and must be submitted to regular decontamination (70% ethanol, 10% solution of bleach, hospital grade disinfectants) of those parts that could accidentally come in contact with the sample or the components of the kit. They should also be regularly maintained in order to show a precision of 1% and a trueness of ±2%.
2. The **ELISA incubator** has to be set at +37°C (tolerance of ±1°C) and regularly checked to ensure the correct temperature is maintained. Both dry incubators and water baths are suitable for the incubations, provided that the instrument is validated for the incubation of ELISA tests.
3. In case of **shaking** during incubations, the instrument has to ensure 350 rpm ±150. Amplitude of shaking is very important as a wrong one could give origin to splashes and therefore to some false positive result.
4. The **ELISA washer** is extremely important to the overall performances of the assay. The washer must be carefully validated and correctly optimized using the kit controls/calibrator and reference panels, before using the kit for routine laboratory tests. Usually 4-5 washing cycles (aspiration + dispensation of 350µl/well of washing solution = 1 cycle) are sufficient to ensure that the assay performs as expected. A soaking time of 20-30 seconds between cycles is suggested. In order to set correctly their number, it is recommended to run an assay with the kit controls/calibrator and well-characterized negative and positive reference samples, and check to match the values reported below in the section "Internal Quality Control". Regular calibration of the volumes delivered and maintenance (decontamination and cleaning of needles) of

the washer has to be carried out according to the instructions of the manufacturer.

5. **Incubation times** have a tolerance of $\pm 5\%$.
6. The **microplate reader** has to be equipped with a reading filter of 450nm and ideally with a second filter (620-630nm) for blanking purposes. Its standard performances should be (a) bandwidth ≤ 10 nm; (b) absorbance range from 0 to ≥ 2.0 ; (c) linearity to ≥ 2.0 ; repeatability $\geq 1\%$. Blanking is carried out on the well identified in the section "Assay Procedure". The optical system of the reader has to be calibrated regularly to ensure that the correct optical density is measured. It should be regularly maintained according to the manufacturer's instructions.
7. When using **ELISA automated workstations**, all critical steps (dispensation, incubation, washing, reading, shaking, data handling, etc.) have to be carefully set, calibrated, controlled and regularly serviced in order to match the values reported in the sections "Internal Quality Control". The assay protocol has to be installed in the operating system of the unit and validated by checking full matching the declared performances of the kit. In addition, the liquid handling part of the station (dispensation and washing) has to be validated and correctly set paying particular attention to avoid carry over by the needles used for dispensing samples and for washing. The carry over effect must be studied and controlled to minimize the possibility of contamination of adjacent wells due to strongly reactive samples, leading to false positive results. The use of ELISA automated work stations is recommended for blood screening and when the number of samples to be tested exceed 20-30 units per run.
8. When using automatic devices, in case the vial holder of the instrument does not fit with the vials supplied in the kit, transfer the solution into appropriate containers and label them with the same label peeled out from the original vial. This operation is important in order to avoid mismatching contents of vials, when transferring them. When the test is over, return the secondary labeled containers to 2.8°C , firmly capped.
9. **Dia.Pro's customer service** offers support to the user in the setting and checking of instruments used in combination with the kit, in order to assure full compliance with the essential requirements of the assay. Support is also provided for the installation of new instruments to be used in combination with the kit.

L. PRE ASSAY CONTROLS AND OPERATIONS

1. Check the expiration date of the kit printed on the external label of the kit box. Do not use if expired.
2. Check that the liquid components are not contaminated by naked-eye visible particles or aggregates. Check that the Chromogen/Substrate is colorless or pale blue by aspirating a small volume of it with a sterile transparent plastic pipette. Check that no breakage occurred in transportation and no spillage of liquid is present inside the box. Check that the aluminum pouch, containing the microplate, is not punctured or damaged.
3. Dilute all the content of the 20x concentrated Wash Solution as described above.
4. Dilute the 20X concentrated Enzyme Conjugate with its Diluent as reported.
5. Dissolve the Calibrator as described above.
6. Allow all the other components to reach room temperature (about 1 hr) and then mix as described.
7. Set the ELISA incubator at $+37^{\circ}\text{C}$ and prepare the ELISA washer by priming with the diluted washing solution, according to the manufacturers instructions. Set the right number of washing cycles as found in the validation of the instrument for its use with the kit.
8. Check that the ELISA reader has been turned on at least 20 minutes before reading.

9. If using an automated workstation, turn it on, check settings and be sure to use the right assay protocol.
10. Check that the micropipettes are set to the required volume.
11. Check that all the other equipment is available and ready to use.
12. In case of problems, do not proceed further with the test and advise the supervisor.

M. ASSAY PROCEDURE

The assay has to be carried out according to what reported below, taking care to maintain the same incubation time for all the samples in testing.

Automated assay:

In case the test is carried out automatically with an ELISA system, we suggest to make the instrument dispense first 150 μl controls & calibrator, then all the samples and finally 100 μl diluted Enzyme Conjugate.

For the pre-washing step (point 1 of the assay procedure) and all the next operations follow the operative instructions reported below for the Manual Assay.

It is strongly recommended to check that the time lap between the dispensation of the first and the last sample will be calculated by the instrument and taken into consideration by delaying the first washing operation accordingly.

Manual Assay:

1. Place the required number of strips in the plastic holder and wash them once to hydrate wells. Carefully identify the wells for controls, calibrator and samples.

Important note: *Pre washing is fundamental to obtain reliable and specific results both in the manual and in the automatic procedures. Do not omit it !*

2. Leave the A1 well empty for blanking purposes.
3. Pipette 150 μl of the Negative Control in triplicate, 150 μl of the Calibrator in duplicate and then 150 μl of the Positive Control in single followed by 150 μl of each of the samples.
4. Check for the presence of samples in wells by naked eye (there is a marked color difference between empty and full wells) or by reading at 450/620nm. (samples show OD values higher than 0.100).
5. Dispense 100 μl diluted Enzymatic Conjugate in all wells, except for A1, used for blanking operations.

Important note: *Be careful not to touch the inner surface of the well with the pipette tip when the conjugate is dispensed. Contamination might occur.*

6. Following addition of the conjugate, check that the color of the samples have changed from yellowish to red and then incubate the microplate for **120 min at $+37^{\circ}\text{C}$** .

Important notes:

- a. *Strips have to be sealed with the adhesive sealing foil, only when the test is performed manually. Do not cover strips when using ELISA automatic instruments.*
- b. *If the procedure is carried out on shaking, be sure to deliver the rpm reported for in Section I.3 as otherwise intra-well contamination could occur.*
7. When the first incubation is over, wash the microwells as previously described (section I.4)
8. Pipette 200 μl Chromogen/Substrate into all the wells, A1 included.

Important note: *Do not expose to strong direct light as a high background might be generated.*

9. Incubate the microplate protected from light at **18-24°C for 30 min.** Wells dispensed with the positive control, the calibrator and positive samples will turn from clear to blue.
10. Pipette 100 µl Sulphuric Acid into all the wells to stop the enzymatic reaction, using the same pipetting sequence as in step 8. Addition of the acid solution will turn the positive control, the calibrator and positive samples from blue to yellow.
11. Measure the color intensity of the solution in each well, as described in section I.6 using a 450nm filter (reading) and if possible a 620-630nm filter (background subtraction), blanking the instrument on A1.

Microplate

	1	2	3	4	5	6	7	8	9	10	11	12
A	BLK	S2										
B	NC	S3										
C	NC	S4										
D	NC	S5										
E	CAL	S6										
F	CAL	S7										
G	PC	S8										
H	S1	S9										

Legenda: BLK = Blank NC = Negative Control
 CAL = Calibrator PC = Positive Control S = Sample

Important notes:

1. If the second filter is not available, ensure that no fingerprints or dust are present on the external bottom of the microwell before reading at 450nm. They could generate false positive results on reading
2. Reading should ideally be performed immediately after the addition of the acid solution but definitely no longer than 20 minutes afterwards. Some self-oxidation of the chromogen can occur leading to a higher background.
3. When samples to be tested are not surely clean or have been stored frozen, the assay procedure reported below is recommended as long as it is far less sensitive to interferences due to hemolysis, hyperlipaemia, bacterial contamination and fibrin microparticles. The assay is carried out in two-steps at +37°C on shaking at 350 rpm ±150 as follows:
 - dispense 100 ul of controls, calibrator and samples
 - incubate 60 min at +37°C on shaking
 - wash according to instructions (section I.4)
 - dispense 100 ul diluted enzyme tracer
 - incubate 30 min at +37°C on shaking
 - wash
 - dispense 100 ul TMB&H2O2 mix
 - incubate 30 min at r.t. on shaking
 - stop and read

In this procedure the pre-wash can be omitted. This method shows performances similar to the standard one and therefore can be used in alternative.

N. ASSAY SCHEME

Operations	Procedure
Pre-Washing step	n° 1 cycle
Controls&Calibrator&samples	150 ul
Diluted Enzyme Conjugate	100 ul
1st incubation	120 min
Temperature	+37°C
Washing steps	n° 4-5
Chromogen/Substrate	200ul
2nd incubation	30 min
Temperature	room
Sulphuric Acid	100 ul
Reading OD	450nm

An example of dispensation scheme is reported in the following section:

O. INTERNAL QUALITY CONTROL

A check is performed on the controls/calibrator any time the kit is used in order to verify whether the expected OD450nm or S/Co values have been matched in the analysis.

Ensure that the following results are met:

Parameter	Requirements
Blank well	< 0.100 OD450nm value
Negative Control (NC)	< 0.050 mean OD450nm value after blanking
Calibrator 0.5 IU/ml (2 nd WHO standard)	S/Co ≥ 2
Positive Control	> 1.000 OD450nm value

If the results of the test match the requirements stated above, proceed to the next section.

If they do not, do not proceed any further and perform the following checks:

Problem	Check
Blank well > 0.100 OD450nm	1. that the Chromogen/Substrate solution has not become contaminated during the assay
Negative Control (NC) > 0.050 OD450nm after blanking	1. that the washing procedure and the washer settings are as validated in the pre qualification study; 2. that the proper washing solution has been used and the washer has been primed with it before use; 3. that no mistake has been done in the assay procedure (dispensation of positive control instead of the negative one); 4. that no contamination of the negative control or of the wells where the control was dispensed has occurred due to spills of positive samples or of the enzyme conjugate; 5. that micropipettes have not become contaminated with positive samples or with the enzyme conjugate 6. that the washer needles are not blocked or partially obstructed.
Calibrator S/Co < 2	1. that the procedure has been correctly performed; 2. that no mistake has occurred during its distribution (ex.:dispensation of negative control instead of calibrator) 3. that the washing procedure and the washer settings are as validated in the pre qualification study; 4. that no external contamination of the calibrator has occurred.
Positive Control < 1.000 OD450nm	1. that the procedure has been correctly performed; 2. that no mistake has occurred during the distribution of the control (dispensation of negative control instead of positive control. In this case, the negative control will have an OD450nm value > 0.050). 3. that the washing procedure and the washer settings are as validated in the pre qualification study; 4. that no external contamination of the positive control has occurred.

If any of the above problems have occurred, report the problem to the supervisor for further actions.

P. CALCULATION OF THE CUT-OFF

The test results are calculated by means of a cut-off value determined on the mean OD450nm value of the negative control (NC) with the following formula:

$$NC + 0.050 = \text{Cut-Off (Co)}$$

The value found for the test is used for the interpretation of results as described in the next paragraph.

Important note: When the calculation of results is performed by the operating system of an ELISA automated work station, ensure that the proper formulation is used to calculate the cut-off value and generate the correct interpretation of results.

Q. INTERPRETATION OF RESULTS

Test results are interpreted as a ratio of the sample OD450nm (S) and the Cut-Off value (Co), mathematically S/Co, according to the following table:

S/Co	Interpretation
< 0.9	Negative
0.9 – 1.1	Equivocal
> 1.1	Positive

A negative result indicates that the patient is not infected by HBV and that the blood unit may be transfused.

Any patient showing an equivocal result should be retested on a second sample taken 1-2 weeks after the initial sample; the blood unit should not be transfused.

A positive result is indicative of HBV infection and therefore the patient should be treated accordingly or the blood unit should be discarded.

Important notes:

1. Interpretation of results should be done under the supervision of the laboratory supervisor to reduce the risk of judgment errors and misinterpretations.
2. Any positive result must be confirmed first by repeating the test on the sample, after having filtered it on 0.2-0.8 µ filter to remove any microparticles interference. Then, if still positive, the sample has to be submitted to a confirmation test before a diagnosis of viral hepatitis is released.
3. When test results are transmitted from the laboratory to another department, attention must be paid to avoid erroneous data transfer.
4. Diagnosis of viral hepatitis infection has to be taken and released to the patient by a suitably qualified medical doctor.

An example of calculation is reported below.

The following data must not be used instead of real figures obtained by the user.

Negative Control: 0.012 – 0.008 – 0.010 OD450nm
Mean Value: 0.010 OD450nm
Lower than 0.050 – Accepted
Positive Control: 2.489 OD450nm
Higher than 1.000 – Accepted
Cut-Off = 0.010 + 0.050 = 0.060
Calibrator: 0.350 - 0.370 OD450nm
Mean value: 0.360 OD450nm S/Co = 6.0
S/Co higher than 2.0 – Accepted
Sample 1: 0.028 OD450nm
Sample 2: 1.690 OD450nm
Sample 1 S/Co < 0.9 = negative
Sample 2 S/Co > 1.1 = positive

R. PERFORMANCE CHARACTERISTICS

Evaluation of Performances has been conducted in accordance to what reported in the Common Technical Specifications or CTS (art. 5, Chapter 3 of IVD Directive 98/79/EC).

1. Limit of detection

The limit of detection of the assay has been calculated by means of the following international preparations:

1. standards for HBsAg - ay and ad subtypes - supplied by Paul Erlich Institute (from preparation 50.000 U/ml and 100 U/ml respectively);
2. NIBSC 2nd British working standard (French/UK standard code 99/640-005-WI)
3. 1st WHO international standard, code n°80/549.
4. 2nd WHO international standard, NIBSC code 00/588.

Results of Quality Control are given in the following table:

Standard Preparation	IU/ml or ng/ml
PEI	
Ad subtype	0.01
Ay subtype	0.01
NIBSC	0.03
WHO 1st standard	0.05
WHO 2nd standard	0.1-0.05

The panel # 806, supplied by Boston Biomedical Inc., USA, was also tested to define the limit of sensitivity.

Results are as follows :

BBI Panel 806

Member ID	HBsAg Subtype	BBI ng/ml	Abbott ng/ml	PEI U/ml	Lot 1 S/Co	Lot 2 S/Co	Lot 3 S/Co
806-1	ad	2,5	3,1	0,31	9.5	9.4	9.7
806-2	ad	1,5	1,6	0,17	5.1	5.0	5.3
806-3	ad	1	1	0,1	3.2	3.2	3.3
806-4	ad	0,8	0,9	0,09	2.6	2.4	2.8
806-5	ad	0,6	0,7	0,07	2.3	2.1	2.4
806-6	ad	0,5	0,5	0,06	2.0	1.8	1.9
806-7	ad	0,4	0,5	0,05	1.7	1.6	1.8
806-8	ad	0,3	0,3	0,03	1.4	1.3	1.5
806-9	ad	0,2	0,2	0,02	1.2	1.1	1.2
806-10	ad	0,1	0,1	0,01	0.8	0.7	0.8

806-11	ay	2,4	1,8	0,21	10.5	10.7	10.6
806-12	ay	1,3	1	0,11	5.3	5.6	5.5
806-13	ay	0,9	0,6	0,07	4.7	4.9	4.9
806-14	ay	0,8	0,5	0,06	3.9	4.0	4.0
806-15	ay	0,6	0,4	0,05	2.6	2.7	2.5
806-16	ay	0,5	0,4	0,04	2.4	2.5	2.5
806-17	ay	0,4	0,3	0,03	1.7	1.8	1.9
806-18	ay	0,3	0,3	0,03	1.6	1.7	1.7
806-19	ay	0,2	0,2	0,02	1.4	1.6	1.5
806-20	ay	0,1	0,1	0,01	1.1	1.2	1.1
806-21	negative	0	0	0	0.2	0.2	0.2

In addition two panels of sensitivity supplied by EFS, France, have been tested and have given the following results:

Sensitivity panel EFS, France

Echantillon	(c) théorique d'Ag HBs ng/ml	Mean OD450nm	S/Co	Ortho S/Co
HB 1	0.10	0.039	0.7	0.6
HB 2	0.20	0.082	1.4	1.2
HB 3	0.30	0.116	2.0	1.8
HB 4	0.45	0.161	2.7	2.8
HB 5	0.66	0.210	3.6	3.9
HB 6	diluent	0.009	0.2	0.2

Sensitivity panel SFTS, France, Ag HBs 2004

Sample ID	Characteristics	ng/ml	S/Co
151	Adw2 + ayw3	2.24 ± 0.15	10.8
152	Adw2 + ayw3	1.15 ± 0.06	5.8
153	Adw2 + ayw3	0.95 ± 0.05	5.0
154	Adw2 + ayw3	0.58 ± 0.04	3.3
155	Adw2 + ayw3	0.50 ± 0.03	2.6
156	Adw2 + ayw3	0.36 ± 0.02	1.9
157	Adw2 + ayw3	0.23 ± 0.02	1.3
158	Adw2 + ayw3	0.13 ± 0.02	0.7
159	Adw2 + ayw3	0.06 ± 0.01	0.4
160	Adw2 + ayw3	0.04 ± 0.01	0.2
161	Adw2	0.5 – 1.0	2.8
162	Adw4	0.5 – 1.0	2.6
163	Adr	0.5 – 1.0	3.0
164	Ayw1	0.5 – 1.0	2.9
165	Ayw2	0.5 – 1.0	3.9
166	Ayw3	0.5 – 1.0	4.6
167	Ayw3	0.5 – 1.0	4.3
168	Ayw4	0.5 – 1.0	4.2
169	Ayr	0.5 – 1.0	3.6
170	diluent	/	0.1

2. Diagnostic sensitivity:

The diagnostic sensitivity has been tested on panels of samples classified positive by a US FDA approved kit.

Positive samples were collected from different geographical regions (including therefore both ad and ay subtypes) and from different HBV pathologies (acute, a-symptomatic and chronic hepatitis B).

An overall value of 100% has been found in the study conducted on a total number of more than 400 samples.

A total of more than 20 sero-conversion and performance panels have also been studied. Results obtained by examining two Panels supplied by Boston Biomedica Inc., USA, are reported below.

BBI PHM 916

Sample ID	HBsAg conc. ng/ml	SAG1. CE S/Co	Ortho EIA S/Co	Sorin EIA S/Co
01	< 0.1	0.4	0.2	0.0
02	< 0.1	0.3	0.2	0.1
03	< 0.1	0.3	0.2	0.0
04	< 0.1	0.4	0.1	0.0
05	< 0.1	0.4	0.2	0.0
06	< 0.1	0.4	0.6	0.1
07	< 0.1	0.4	0.2	0.1
08	< 0.1	0.5	0.2	0.1
09	0.2	0.9	0.4	0.2
10	0.5	1.4	2.2	1.2
11	2.3	4.5	10.1	6.2

BBI PHM 923

Sample ID	HBsAg conc. ng/ml	SAG1. CE S/Co	Ortho EIA S/Co	Sorin EIA S/Co
01	< 0.1	0.4	0.1	0.0
02	< 0.1	0.4	0.2	0.1
03	< 0.2	1.2	1.4	0.9
04	< 0.5	1.4	2.6	1.8

3. Diagnostic specificity:

The diagnostic specificity has been determined on panels of negative samples from normal individuals and blood donors, classified negative with a kit registered in Italy by the Ministry of Health.

Both plasma, derived with different standard techniques of preparation (citrate, EDTA and heparin), and sera have been used to determine the specificity.

No false reactivity due to the method of specimen preparation has been observed.

Frozen specimens have also been tested to check whether samples freezing interferes with the performance of the test. No interference was observed on clean and particle free samples.

All the HBsAg known subtypes, "ay" and "ad", and isoforms "w" and "r", supplied by CNTS, France, were tested in the assay and determined positive by the kit as expected.

Samples derived from patients with different viral (HCV, HAV) and non viral pathologies of the liver that may interfere with the test were examined.

No cross reaction were observed.

The Performance Evaluation study conducted in a qualified external reference center on more than 5000 samples has provided a value of > 99.5% .

4. Precision:

It has been calculated on three samples examined in replicates in different runs. The mean values obtained from a study conducted on two samples of different HBsAg reactivity, examined in 16 replicates in three separate runs (Standard Procedure) for three lots is reported below:

Lot # 1

Negative Sample (N = 16)

Mean values	1st run	2nd run	3 rd run	Average
OD 450nm	0.036	0.036	0.032	0.034
Std.Deviation	0.004	0.006	0.005	0.005
CV %	10.1	15.5	16.2	13.9

NIBSC 0.1 ng/ml (N = 16)

Mean values	1st run	2nd run	3 rd run	Average
OD 450nm	0.133	0.121	0.118	0.124
Std.Deviation	0.019	0.008	0.011	0.013
CV %	14.1	6.9	9.2	10.1
S/Co	1.5	1.4	1.4	1.4

Lot # 2

Negative Sample (N = 16)

Mean values	1st run	2nd run	3 rd run	Average
OD 450nm	0.034	0.035	0.031	0.033
Std.Deviation	0.005	0.005	0.005	0.005
CV %	15.5	13.5	15.4	14.8

NIBSC 0.1 ng/ml (N = 16)

Mean values	1st run	2nd run	3 rd run	Average
OD 450nm	0.125	0.129	0.129	0.127
Std.Deviation	0.012	0.010	0.013	0.012
CV %	9.7	7.5	10.4	9.2
S/Co	1.5	1.5	1.6	1.5

Lot # 3

Negative Sample (N = 16)

Mean values	1st run	2nd run	3 rd run	Average
OD 450nm	0.034	0.035	0.034	0.034
Std.Deviation	0.004	0.005	0.004	0.004
CV %	10.5	12.9	11.2	11.5

NIBSC 0.1 ng/ml (N = 16)

Mean values	1st run	2nd run	3 rd run	Average
OD 450nm	0.124	0.129	0.126	0.126
Std.Deviation	0.009	0.012	0.015	0.012
CV %	7.2	9.4	11.7	9.4
S/Co	1.5	1.5	1.5	1.5

The variability shown in the tables did not result in sample misclassification.

S. LIMITATIONS

Repeatable false positive results were assessed on freshly collected specimens in less than 0.1% of the normal population, mostly due to high titers Heterophilic Anti Mouse Antibodies (HAMA). Interferences in fresh samples were also observed when they were not clean or badly collected (see chapter G). Frozen samples, presenting fibrin particles, crioglobulins, lipid-containing micelles or microparticles after thawing, generated some false positive results.

Interferences were almost totally abolished when the same samples were tested with the alternative two-steps method.

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<p>This product has been manufactured by Dia. Pro s.r.l. under the controls established by a quality management system that meets the requirements of EN ISO 13485 as assessed by the EC Notified Body n°0318</p>
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