

Instructions for use

For *in vitro* diagnostic use only

HBsAg EIA Plus

A solid-phase enzyme immunoassay for the detection of Hepatitis B Surface Antigen (HBsAg) in human serum or plasma.

Product no. 61 10 800
(Microstrips®, 96 wells)

Product no. 61 10 802
(Microstrips®, 480 wells)

HBsAg Confirmatory Test

For confirmation of HBsAg reactive specimens by neutralization.

Product no. 61 06 070

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INTENDED USE

Ani Labsystems' HBsAg EIA Plus is an *in vitro* enzyme immunoassay for the detection of hepatitis B surface antigen in human serum or plasma.

INTRODUCTION

Hepatitis B infection is highly endemic in South-East Asia and Africa and its prevalence in industrialized countries is

high among IV-drug abusers, homosexuals and prison inmates. Of the infected people 1-5 % will remain as chronic carriers of hepatitis B virus. Major part of the chronic carriers secrete hepatitis B surface antigen (HBsAg) into blood and other secretions of the body like saliva and vaginal fluid. These chronic carriers are potentially infectious to other seronegative people. Minority of the HBsAg carriers have the e-antigen (HBeAg) as a sign of an active hepatitis B infection. A sample containing HBeAg is about 10⁵-10⁶ times more infectious than a sample which is HBsAg positive but HBeAg negative.

HBsAg presents an antigenic heterogeneity. The principal determinant is called *a* and it is common to all different types of HBsAg. There are two other pairs of major determinants, that is *d/y* and *w/r*, which are mutually exclusive. The following combinations are possible: *adw*, *adr*, *ayw* and *ayr*.

The HBsAg EIA Plus is primarily used for testing blood and organ donors, patients with active viral hepatitis and people at risk for hepatitis B infection.

PRINCIPLE OF THE TEST

The principle of Ani Labsystems' HBsAg EIA Plus is based on a direct, non-competitive solid-phase enzyme immunoassay with horseradish peroxidase as the marker enzyme (1). The assay proceeds according to the following reactions.

1. When present in patient serum HBsAg (●) will combine with the mouse monoclonal anti-HBs (↵)

attached to the polystyrene surface (|) of the Microstrips® and simultaneously bind with the horseradish peroxidase conjugated sheep poly- and mouse monoclonal anti-HBs (⌘).



2. Wells are washed and a colourless enzyme substrate (H₂O₂) and chromogen (TMB*) are added. The enzyme action on substrate/chromogen produces a coloured end product.



3. The enzyme-substrate/chromogen reaction is terminated with acid (H₂SO₄). The colour intensity is directly related to the concentration of hepatitis B surface antigen in the patient sample.

* Tetramethylbenzidine (2, 3), a non-mutagenic chromogen for horseradish peroxidase.

KIT CONTENTS

- NOTE:**
- For *in vitro* diagnostic use only.
 - Wear disposable gloves while handling specimens and kit reagents. Afterwards wash hands carefully.
 - Reagents are sufficient for 96 or 480 wells. The amounts of reagents for 480 wells kit size are indicated below in the parenthesis.
 - Reagents are stored between +2°C and +8°C.
 - **Prewarm all reagents and Microstrips® to +20°C - +25°C before use.**
 - The expiration date is printed on each component and on the package.
 - Avoid excessive exposure to light. This is merely a precaution. The light sensitive reagents are the chromogen, the conjugate and the substrate buffer.
 - Once opened, the components must be sealed tightly!
 - Once the Microstrip® foil-package has been opened it has to be resealed tightly and stored at +2°C to +8°C with desiccant.
 - Before use mix well the reagents by inverting the vials.

1 Anti-HBs COATED MICROSTRIPS®, 12 x 8 wells (5 plates of 12 x 8 wells)
Microstrips® coated with anti-HBs antibodies (mouse, monoclonal), having the same reactivity for *ad* and *ay* subtypes.

2a NEGATIVE CONTROL, 2.5 ml (5 ml)
Diluted foetal bovine serum, proprietary additives and 0.05 % Bronidox® as preservative.

2b POSITIVE CONTROL, 2.5 ml (5 ml)
Recombinant HBsAg/*ay* (approx. 1 U/ml *) produced in a Vero-hepatocyte hybrid cell line, proprietary additives and 0.05 % Bronidox® as preservative.

* Calibrated against the Paul Ehrlich Institute (Frankfurt am Main, Germany) HBsAg/*ay* standard preparation.

3 Anti-HBs-HRP-CONJUGATE (SHEEP AND MOUSE), 20 ml (3 x 20 ml)
Horseradish peroxidase conjugated anti-HBs antibodies (sheep and mouse), proprietary additives, a blue colouring reagent and 0.01 % Thiomersal® as preservative.

4 SUBSTRATE BUFFER, 50 ml (100 ml)
Citrate-asetate buffer containing 0.005 % hydrogen peroxide and 0.05 % Bromo-Nitro-Dioxane as preservative.

- 5** TMB-CHROMOGEN IN DMSO, 1 ml (3 ml)
3,3', 5,5'-Tetramethylbenzidine dissolved in dimethyl sulfoxide (DMSO). Precautions on page 3. R36/38, S26
- 6** WASHING SOLUTION, 100 ml concentrate (3 x 100 ml concentrate)
Concentrated citrate buffered saline, proprietary additives and 0.05 % Bronidox® as preservative.

MICROSTRIP® COVERS, 2 pcs (10 pcs)
Plastic sheets to cover the Microstrips® during incubation.

REAGENT PREPARATION

Reagent	Preparation	Stability of opened/ diluted reagents (+2°C to +8°C)
1 Anti-HBs coated Microstrips®	Ready for use	6 months *)
2a Negative control	Ready for use	6 months *)
2b Positive control	Ready for use	6 months *)
3 Anti-HBs-HRP Conjugate	Ready for use	6 months *)
4 Substrate buffer	Ready for use for the dilution of TMB-Chromogen	6 months *)
5 TMB-Chromogen	Dilute 1 + 49 (1:50) with substrate buffer (vial 4) just before use	Discard unused solution. A deep blue colour present in the substrate solution indicates that the solution has been contaminated and must be discarded.
6 Washing solution	Dilute 1 + 9 (1:10) with distilled water	1 week
Stopping solution **)	Ready for use	

*) The stability of the opened reagents is maximum 6 months only if they are stored properly at +2°C to +8°C. However, high environmental temperature and contamination may decrease the stability.

***) Not included in kit composition. Available from Ani LabSystems - refer to section MATERIALS REQUIRED BUT NOT PROVIDED.

MATERIALS REQUIRED BUT NOT PROVIDED

- Distilled or deionized water, preferably sterile.
- Graduated cylinders for reagent dilutions.
- Vials to store the diluted reagents.
- Precision pipettes (e.g.: one channel 0.5-10 μ l, 5-50 μ l, 20-200 μ l, 200-1000 μ l, and multi channel 50-300 μ l.)
- Paper towels or absorbent paper.
- Timer.
- Microplate incubator/Shaker, +37°C, 900 rpm.
- Microplate photometer (plate or Microstrip® reader), 450 nm.
- Microplate washer.
- Sodium hypochlorite solution (free available chlorine 50-500 mg/l) (4).
- Disposable gloves.
- Stopping solution (0.45 M H₂SO₄). Available from Ani Lab systems (0.45 M sulphuric acid, 15 ml - cat. no. 61 10 366).

SPECIMEN COLLECTION AND HANDLING

Specimens must be centrifuged before use (e.g. 3000 RPM, 10 minutes), especially citrate plasma specimens will cause false HBsAg reactive results, if not centrifuged properly.

Serum and plasma (preferably EDTA) samples may be stored for up to 7 days at +2°C to +8°C or at least 6 months as frozen (-20°C or -70°C). Samples should not be repeatedly frozen and thawed.

Do not use heat inactivated samples. Especially heat inactivated plasma specimens will cause false HBsAg reactive results.

Do not use sodium azide as preservative because it inactivates horseradish peroxidase.

Microbially-contaminated, grossly hemolyzed, icteric or hyperlipemic serum and plasma specimens may give erroneous results.

PRECAUTIONS

For *in vitro* diagnostic use only.

The positive control contains recombinant HBsAg produced in a Vero-hepatocyte hybrid cell line in which no infectious hepatitis B virus could be detected.

The negative control contains foetal bovine serum in which no infectious hepatitis B virus could be detected.

All human serum and plasma samples should be considered potentially infectious. It is recommended that all specimens of human origin should be handled at the Biosafety Level 2 as recommended for any potentially infectious human serum or blood specimen in the Centers

for Disease Control/National Institute of Health manual "Biosafety in Microbiological and Biomedical Laboratories", 1984.

Discard all materials and specimens as if capable of transmitting infection (4, 5, 6, 7). The preferred method of disposal is autoclaving for a minimum of one hour at 121°C. Liquid wastes **not containing** acid may be mixed with sodium hypochlorite in volumes such that the final mixture contains 50-500 mg/l available chlorine. Allow 30 minutes for decontamination to be completed.

NOTE: Liquid waste containing acid must be neutralized with a proportional amount of base prior to the addition of sodium hypochlorite.

Spills should be wiped up thoroughly using either an iodophor disinfectant or sodium hypochlorite solution. Materials used to wipe up spills should be added to biohazardous waste matter for proper disposal (4, 5, 6, 7).

Wear disposable gloves while handling specimens and kit reagents. Afterwards wash hands carefully. Never pipette by mouth. Avoid splashing or forming aerosols.

Deterioration is indicated by a significant decrease in the absorbance level of positive control.

Stopping solution (cat. no. 6110 366) contains 0.45 M (4.4%) sulphuric acid. Avoid contact with skin and eyes.

The TMB-chromogen is diluted in dimethyl sulfoxide (DMSO). Avoid contact with skin and eyes.

The melting point of DMSO is +18°C. Avoid exposure of TMB solution to intense source of light. Oxidizing agents, metallic ions or soap remaining in glassware containers can interfere with the TMB reaction. In order to avoid this problem rinse the glassware thoroughly with 1 N acid (HCl or H₂SO₄) followed by several washes with distilled water before use.

Reagents are stored between +2°C and +8°C. Avoid unnecessary exposure to light. This is merely a precaution. The light sensitive reagents are the chromogen, the conjugate and the substrate buffer.

Do not use reagents after the expiration date printed on the label.

Do not mix or interchange reagents from different lots. Do not interchange vial caps.

When removing aliquots from the reagent vials, use aseptic technique to avoid contamination or incorrect results may occur. Use a new pipette tip for each sample. Do not touch the wells or splash the wells while pipetting. Reusable glassware must be disinfected, washed out and rinsed free of detergents.

Accurate and precise pipetting, as well as following the exact time and temperature requirements, is essential.

Once the assay has been started, all steps should be performed without interruption. Do not let wells dry once the assay has started.

Do not reuse a Microstrip® even though some wells were not used in the assay.

TEST PROCEDURE

Outline of the Procedure

- STEP I** Add 50 µl conjugate
- Add 100 µl controls/specimens
- Incubate and shake 120 min., +37°C (Procedure A)
Incubate 16-22 h, RT (Procedure B)
Incubate 2-3 h, +37°C (Procedure C)
Incubate 1h, +37°C (Procedure D)
- Wash 5x
- STEP II** Add 100 µl substrate solution
- Incubate 30 min., RT in a dark place
- STEP III** Add 100 µl 0.45 M H₂SO₄
- Measure at 450 nm

Preliminary Preparations

NOTE: Wear disposable gloves throughout the procedure!

Bring all reagents and Microstrips® to room temperature (+20 to +25°C) before starting the assay.

Prewarm the incubator to +37°C when performing procedure A, C or D.

Prepare the washing solution and bring to room temperature.

Test Procedure

- STEP I**
1. Pipette 50 µl of conjugate (vial 3) into the bottom of Microstrip® wells.
 2. Pipette 100 µl of the negative control (vial 2a) into three wells (A1, B1, C1).
 3. Pipette 100 µl of the positive control (vial 2b) into two wells (D1, E1).
 4. Pipette 100 µl of specimens into the Microstrip® wells, starting from well F1. Cover the Microstrip® wells with Microstrip® cover.
 5. Incubate according to the procedure chosen:

- Procedure A:** 120 minutes at +37°C with continuous shaking (900 rpm).
- Procedure B:** Static incubation 16-22 hours at room temperature.
- Procedure C:** Static incubation 2-3 hours at +37°C.
- Procedure D:** Static incubation 1 hour at +37°C.

WASHING

Washing can be performed either with a washer, or manually.

6. Empty the wells.
7. Add 300-400 µl of washing solution into each well. Soak at least 10 seconds.
8. Empty the wells.
9. Wash each well **five** times in total.
10. After the fifth wash, tap the inverted Microstrips® a few times on a paper towel.

STEP II

1. Add 100 µl of substrate solution (vials 4 and 5) into each well.
Avoid contamination of substrate solution: do not touch the walls of the wells with pipette tips when adding substrate.
Do not cover the Microstrips® with a plastic sheet.
2. Incubate for 30 minutes at room temperature in a dark place.

STEP III

1. Stop the enzyme-substrate reaction by adding 100 µl of 0.45 M H₂SO₄-solution into each well.

NOTE: The use of an 8-channel multipipetting device will minimize the time factor between Microstrips® and improve results when adding the substrate and stopping solution.

Reading of the Results

Blank the photometer at 450 nm against air (no plate in carriage) and measure the absorbances within 30 minutes after stopping the enzyme-substrate reaction.

RESULTS

Quality Control Values

NEGATIVE CONTROL MEAN (NCx)

Individual negative control values should be less than or equal to 0.150 when the photometer is blanked against air, greater than or equal to 0.5 times NCx and less than or equal to 1.5 times NCx. If one of the values is outside the acceptable range, discard this value and recalculate the mean. If two of the values are outside the range, the test should be repeated.

POSITIVE CONTROL MEAN (PCx)

PCx value has been defined based on lot to lot follow-up. To achieve the expected detection limit with Procedure A and B the value of PCx minus NCx should be greater than or equal to 0.900. For procedure C and D, PCx minus NCx should be greater than or equal to 0.400 and 0.200, respectively. If not, the technique may be suspected and the run should be repeated.

Calculation of the Results

CALCULATION OF THE NEGATIVE CONTROL MEAN (NCx)

Determine the mean of the negative control values (Procedure A).

Example:

Negative control Sample no.	Absorbance
1	0.054
2	0.048
3	0.048
Total	0.150

$$NCx = \frac{\text{Total absorbance}}{3} = \frac{0.150}{3} = 0.050$$

In the example, all negative control values are inside the quality control range and the NCx needs not to be revised.

CALCULATION OF THE POSITIVE CONTROL MEAN (PCx)

Determine the mean of the positive control values (Procedure A).

Example:

Positive Control Sample no.	Absorbance
1	1.380
2	1.290
Total	2.670

$$PCx = \frac{\text{Total absorbance}}{2} = \frac{2.670}{2} = 1.335$$

$$PCx - NCx = 1.335 - 0.050 = 1.285$$

In the example PCx minus NCx is greater than 0.900, thus the technique is acceptable and data should be considered valid.

CALCULATION OF THE CUT-OFF VALUE (CO)

Determine the cut-off value by adding 0.05 to the negative control mean (NCx) for **Procedures A, B** and **C**. This cut-off value is used to achieve the highest possible sensitivity eg. in blood donor centers.

$$CO = NCx + 0.050$$

Example: $CO = 0.050 + 0.05 = 0.100$

Alternatively a higher cut-off value may be used eg. in clinical laboratories.

$$CO = NCx + 0.100$$

Example: $CO = 0.050 + 0.1 = 0.150$

Determine the cut-off value by adding 0.025 to the negative control mean (NCx) for **Procedure D**.

$$CO = NCx + 0.025$$

Example: $CO = 0.050 + 0.025 = 0.075$

Results

- a) **Non-reactive:**
A test sample is considered to be non-reactive for HBsAg if the resulting absorbance value is less than the cut-off value.
- b) **Reactive:**
A test sample is considered to be reactive for HBsAg if the resulting absorbance value is greater than or equal to the cut-off value.

Interpretation of the Results

A non-reactive result means that the sample tested either contains no HBsAg or contains HBsAg below the detection limit of Ani Lab systems' HBsAg EIA Plus.

A test sample giving a reactive result has to be retested in duplicate. If either of the duplicates gives a positive result, the sample is considered repeatedly reactive. If both of the duplicates give negative values, the sample is considered to be non-reactive for HBsAg. All highly sensitive immunoassay systems have a potential for nonspecific reactions and therefore all repeatedly reactive specimens should be confirmed by a neutralization assay using the **Procedure A, B or C** (Ani Lab systems' HBsAg Confirmatory Test - cat. no. 61 06 070).

A repeatedly reactive specimen, confirmed reactive by a neutralization assay must be considered positive for HBsAg.

As in any diagnostic enzyme immunoassay, there is a possibility that non-repeatable reactions may occur for the following reasons:

- Inadequate washing
- Contamination of reaction well with anti-HBs-HRP conjugate
- Contamination of substrate solution with conjugate or with oxidizing agents
- Cross-contamination of non-reactive specimens by HBsAg.

PERFORMANCE CHARACTERISTICS

Accuracy

Ani LabSystems' HBsAg EIA Plus (Procedure A, B and C) meets the requirements for a third generation test when tested against the Food and Drug Administration (FDA) HBsAg reference panel no. 6 (FDA Office of Biologics, Bethesda, MD).

Subtype specificity of Ani LabSystems' HBsAg EIA Plus was tested with the HBsAg subtype panel (Centre National de Transfusion Sanquine Institut, Paris, France) consisting of the following HBsAg subtypes: *ayw₁*, *ayw₂*, *ayw₃*, *ayw₄*, *ayr*, *adw₂*, *adw₄*, *adrq⁺*, *adr* (*q* neg) and *ayw₃* * (intermediate between *ayw₃* and *ayw₄*). All of these subtypes were clearly HBsAg reactive with Ani LabSystems' HBsAg EIA Plus and were confirmed as HBsAg positive with Ani LabSystems' HBsAg Confirmatory Test.

Precision

The intra-assay variation of Ani LabSystems' HBsAg EIA Plus was determined by testing one negative (foetal bovine serum, FCS) and two positive samples containing HBsAg/ay 6.5 - 0.09 U/ml. Each sample was tested as 20 parallels (negative and positive control of the test kit as 4 parallels) with Procedure A, B, C and D. Operator-to-operator variation was calculated from the results of intra-assay variation study performed by three technicians. Summary of the results is presented in Table 1.

Table 1. Summary of the intra-assay and operator-to-operator variation study of Ani LabSystems' HBsAg EIA Plus

	Intra-assay variation				Operator-to-operator variation		
	n	Mean ($A_{450\text{ nm}}$)	SD	CV%	Mean ($A_{450\text{ nm}}$)	SD	CV%
Procedure A							
NC	4	0.040	0.001	2.5	0.041	0.002	4.9
PC	4	1.301	0.042	3.2	1.381	0.073	5.3
FCS	20	0.043	0.002	4.7	0.044	0.002	4.5
0.9 U/ml	20	1.455	0.038	2.6	1.454	0.005	0.3
0.09 U/ml	20	0.211	0.004	1.9	0.215	0.004	1.9
Procedure B							
NC	4	0.047	0.002	4.2	0.049	0.009	18.4
PC	4	1.803	0.103	5.7	1.669	0.116	7.0
FCS	20	0.047	0.002	4.3	0.051	0.010	19.6
0.9 U/ml	20	1.160	0.029	2.5	1.098	0.062	5.6
0.09 U/ml	20	0.166	0.004	2.4	0.170	0.016	9.4
Procedure C (2 h at +37°C)							
NC	4	0.038	0.001	2.5	0.040	0.002	3.8
PC	4	0.609	0.028	4.6	0.724	0.142	19.5
FCS	20	0.041	0.002	4.7	0.040	0.003	7.3
1.4 U/ml	20	0.699	0.028	4.0	0.696	0.047	6.7
0.16 U/ml	20	0.114	0.006	5.3	0.116	0.012	10.6
Procedure C (3h at +37°C)							
NC	4	0.040	0.002	5.2	0.046	0.010	21.7
PC	4	1.033	0.026	4.2	1.061	0.041	3.9
FCS	20	0.043	0.001	3.5	0.048	0.010	19.8
0.9 U/ml	20	0.747	0.022	2.9	0.739	0.012	1.6
0.09 U/ml	20	0.114	0.004	3.7	0.125	0.012	9.5
Procedure D (1h at +37°C)							
NC	4	0.050	0.002	5.0	0.054	0.005	8.5
PC	4	0.395	0.004	1.0	0.404	0.008	2.0
FCS	20	0.047	0.002	4.1	0.050	0.004	7.3
6.5 U/ml	20	1.186	0.031	2.6	1.140	0.054	4.7
0.8 U/ml	20	0.228	0.005	2.2	0.223	0.008	3.8

The interassay variation of Ani LabSystems' HBsAg EIA Plus was determined by testing one negative (foetal bovine serum, FCS) and two positive samples containing HBsAg/ay 6.5 - 0.09 U/ml in 10 independent test runs. Each sample was tested as 8 parallels with Procedure A,

B, C and D. Negative and positive control of the test kit was tested in all runs as 4 parallels. Summary of the results is presented in Table 2.

Table 2. Summary of the interassay variation study of Ani LabSystems' HBsAg EIA Plus.

	Procedure A				Procedure B		
	Mean ($A_{450\text{ nm}}$)	SD	CV %		Mean ($A_{450\text{ nm}}$)	SD	CV %
NC	0.039	0.002	5.1	NC	0.043	0.003	7.0
PC	1.302	0.033	2.5	PC	1.686	0.072	4.3
FCS	0.042	0.002	4.8	FCS	0.043	0.002	4.7
0.9 U/ml	1.395	0.047	3.4	0.9 U/ml	1.132	0.022	1.9
0.09 U/ml	0.208	0.005	2.4	0.09 U/ml	0.163	0.004	2.5
	Procedure C (2 h +37°C)				Procedure D (1 h +37°C)		
	Mean ($A_{450\text{ nm}}$)	SD	CV %		Mean ($A_{450\text{ nm}}$)	SD	CV %
NC	0.049	0.003	6.8	NC	0.056	0.004	7.5
PC	0.834	0.033	3.9	PC	0.393	0.022	5.6
FCS	0.048	0.003	6.4	FCS	0.054	0.003	6.2
1.4 U/ml	0.781	0.054	7.0	6.5 U/ml	1.162	0.046	4.0
0.16 U/ml	0.127	0.006	5.0	0.8 U/ml	0.222	0.007	3.1

Specificity

The percentage of specimens found to be reactive with Ani LabSystems' HBsAg EIA Plus (Procedure A, B, C and D) with low and high cut-off value was determined by testing plasma specimens from Finnish blood donors

and serum samples from Finnish and German hospital laboratories. The presence of repeatedly HBsAg reactive specimen was confirmed with Ani LabSystems' HBsAg Confirmatory Test. The summary of the results is presented in Table 3.

Table 3. Summary of the specificity studies of Ani LabSystems' HBsAg EIA Plus.

	Population group	Nonreactive	Repeatedly reactive	Confirmed reactive	Positive
Procedure A CO=NCx+0.05	Blood donors	629 (99.68%)	2 (0.32%)	1 (0.16%)	0 (0%)
	Finnish hospital laboratories	793 (98.27%)	14 (1.73%)	7 (0.87%)	5 *) (0.62%)
	German hospital laboratories	2059 (98.23%)	37 (1.77%)	-	23 (1.10%)
Procedure A CO=NCx+0.1	Blood donors	631 (100%)	0 (0%)	0 (0%)	0 (0%)
	Finnish hospital laboratories	796 (98.64%)	11 (1.36%)	6 (0.74%)	4 (0.50%)
	German hospital laboratories	2068 (98.66%)	28 (1.34%)	-	22 (1.05%)
Procedure B CO=NCx+0.05	Blood donors	628 (99.52%)	3 **)	2 (0.32%)	0 (0%)
	Finnish hospital laboratories	792 (98.14%)	15 (1.86%)	6 (0.74%)	3 (0.37%)
Procedure B CO=NCx+0.1	Blood donors	629 (99.68%)	2 (0.32%)	2 (0.32%)	0 (0%)
	Finnish hospital laboratories	797 (98.76%)	10 (1.24%)	4 (0.50%)	3 (0.37%)
Procedure C (2h at +37°C) CO=NCx+0.05	Blood donors	256 (100%)	0 (0%)	0 (0%)	0 (0%)
	Finnish hospital laboratories	250 (97.66%)	6 (2.34%)	6 (2.34%)	5 (1.95%)
Procedure D (1h at +37°C) CO=NCx+0.025	Blood donors	256 (100%)	0 (0%)	0 (0%)	0 (0%)
	Finnish hospital laboratories	253 (99.22%)	2 (0.78%)	0 (0%)	0 (0%)

*) One of the repeatedly reactive samples was considered to be suspect HBsAg positive with Ani LabSystems' HBsAg Confirmatory Test.

**) One of the initially reactive sample was not retested because lack of the specimen.

Sensitivity

The detection limit of Ani LabSystems' HBsAg EIA Plus (Table 4) was determined using the following standard preparations as references:

A: The Paul Ehrlich Institute (PEI; Frankfurt am Main, Germany) HBsAg Reference Antigen 87 (heat inactivated HBsAg, subtypes *ay* and *ad*, 1000 U/ml).

B: The WHO 1st International Standard (1985; National Institute for Biological Standards and Control, Herfordshire, UK) for HBsAg (HBsAg subtype *ad*, 100 IU/ampoule).

C: Boston Biomedica, Inc. (BBI; W. Bridgewater, MA) HBsAg sensitivity Panel (Lot PHA 802, concentration of HBsAg subtype *ay* and *ad* from 2.2 to 0.1 ng/ml).

Table 4. The detection limit of Ani LabSystems' HBsAg EIA Plus with Procedure A, B, C (low cut-off value) and D was estimated to be as follows:

Reference	Procedure A and B	Procedure C	Procedure D
PEI, subtype ay	0.03 - 0.04 U/ml	0.07 - 0.15 U/ml	0.1 - 0.2 U/ml
subtype ad	0.03 - 0.04 U/ml	0.07 - 0.20 U/ml	0.1 - 0.2 U/ml
WHO, subtype ay	0.05 - 0.10 IU/ml	0.20 - 0.40 IU/ml	0.20 - 0.40 IU/ml
BBI, subtype ay	0.3 - 0.4 ng/ml	1.1 - 1.4 ng/ml	1.3 - 2.4 ng/ml
subtype ad	0.3 - 0.4 ng/ml	1.1 - 1.4 ng/ml	1.4 - 2.5 ng/ml

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HBsAg Confirmatory Test

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- The expiration date is printed on each component and on the package.
- Once opened, the components must be sealed tightly.
- Before use mix well the reagents by inverting the vials.

- 1 CONFIRMATORY (NEUTRALIZATION) REAGENT, 1.5 ml**
Antibodies (rabbit) to hepatitis B surface antigen, 0.05 % Bronidox® as preservative.
- 2 CONTROL REAGENT, 1.5 ml**
Normal rabbit serum, 0.05 % Bronidox® as preservative.
- 3 SAMPLE DILUENT, 2 x 90 ml**
Phosphate buffered saline, proprietary additives and 0.05 % Bronidox® as preservative.

INTENDED USE

Ani LabSystems' HBsAg Confirmatory Test is a neutralization assay to confirm the presence of HBsAg in human serum or plasma.

PRINCIPLE OF THE TEST

A specimen which is found to be repeatedly reactive with Ani LabSystems' HBsAg EIA Plus should be confirmed with Ani LabSystems HBsAg Confirmatory Test. The assay uses the principle of specific antibody neutralization to confirm the presence of HBsAg. The confirmatory reagent (rabbit antibodies to hepatitis B surface antigen) is incubated with the specimen and horseradish peroxidase conjugated anti-HBs (sheep and mouse) in solution. The neutralized HBsAg is subsequently blocked from binding to the conjugate or to the antibody coated Microstrips®. This results in a reduction of signal when compared to the non-neutralized specimen (control) in which control reagent (normal rabbit serum) is used in place of confirmatory reagent.

A specimen is confirmed as HBsAg positive if the reduction in signal of the neutralized specimen is at least 50 % and the non-neutralized specimen generates a signal greater than or equal to the assay cut-off.

KIT CONTENTS

- NOTE:**
- For *in vitro* diagnostic use only.
 - Wear disposable gloves while handling specimens and kit reagents. Afterwards wash hands carefully.
 - Reagents are sufficient for confirmation of 30 specimens/controls as HBsAg positive or negative when used in manual procedure.
 - Reagents are stored between +2°C and +8°C.
 - **Prewarm all reagents and Microstrips® to +20°C - +25°C before use.**

REAGENT PREPARATION

Reagent	Preparation	Stability of opened reagents (+2°C to +8°C)
1 Confirmatory reagent	Ready for use	6 months *)
2 Control reagent	Ready for use	6 months *)
3 Sample diluent	Ready for use	6 months *)

*) The stability of the opened reagents is maximum 6 months only if they are stored properly at +2°C to +8°C. However, high environmental temperature and contamination may decrease the stability.

MATERIALS REQUIRED BUT NOT PROVIDED

Ani LabSystems' HBsAg EIA Plus kit (cat. no. 61 10 800). Please also refer to page 3.

SPECIMEN COLLECTION AND HANDLING

Please refer to page 3.

PRECAUTIONS

Please refer to pages 3 - 4.

TEST PROCEDURE

Outline of the Procedure

- STEP I** Add 25 μ l confirmatory/control reagent
- Add 50 μ l conjugate solution
- Add 100 μ l controls/specimens
- Incubate and shake 120 min., +37°C (Procedure A)
 Incubate 16-22 h, RT (Procedure B)
 Incubate 2-3 h, +37°C (Procedure C)
- Wash 5x
- STEP II** Add 100 μ l substrate solution
- Incubate 30 min., RT in a dark place
- STEP III** Add 100 μ l 0.45 M H₂SO₄
- Measure at 450 nm

Preliminary Preparations

NOTE: Wear disposable gloves throughout the procedure!

Bring all HBsAg EIA Plus and HBsAg Confirmatory Test kit reagents to room temperature (+20 to +25°C) before starting the assay.

Prewarm the incubator to +37°C when procedure A or C is used.

Specimen Dilution

Dilute specimens 1:100 with sample diluent (10 μ l serum or plasma ad 1.0 ml of diluent). Mix well.

For each unknown specimen two undiluted and two diluted (1:100) samples are run; one undiluted and one diluted (1:100) to be neutralized, one undiluted and one diluted (1:100) as non-neutralized controls.

NOTE: It is important to dilute (1:100) also specimens representing the absorbance value < 2.000, because in cases of very high HBsAg concentrations the hook effect observed in one-step HBsAg assays may decrease the absorbances obtained to the level (< 2.000) also seen when low positive specimens are tested. In these situations the confirmatory reagent is not sufficient to neutralize such a high amount of HBsAg. This may lead to wrong interpretation of the HBsAg Confirmatory Test and thus the dilution of all samples is necessary.

Sometimes high titer HBsAg specimens will not be neutralized 50 % by addition of confirmatory reagent when tested undiluted or even diluted 1:100. These specimens should be diluted 1:250 or more with sample diluent and retested with the HBsAg Confirmatory Test.

Controls

Four replicates of HBsAg negative and positive controls must be assayed with each run. Of the four replicates, two will be neutralized and two non-neutralized controls.

NOTE: DO NOT DILUTE THE CONTROLS.

Procedure Selection

Depending on the Ani LabSystems' HBsAg EIA Plus procedure that has been used to screen the specimen, the HBsAg Confirmatory Test procedure must be chosen as follows:

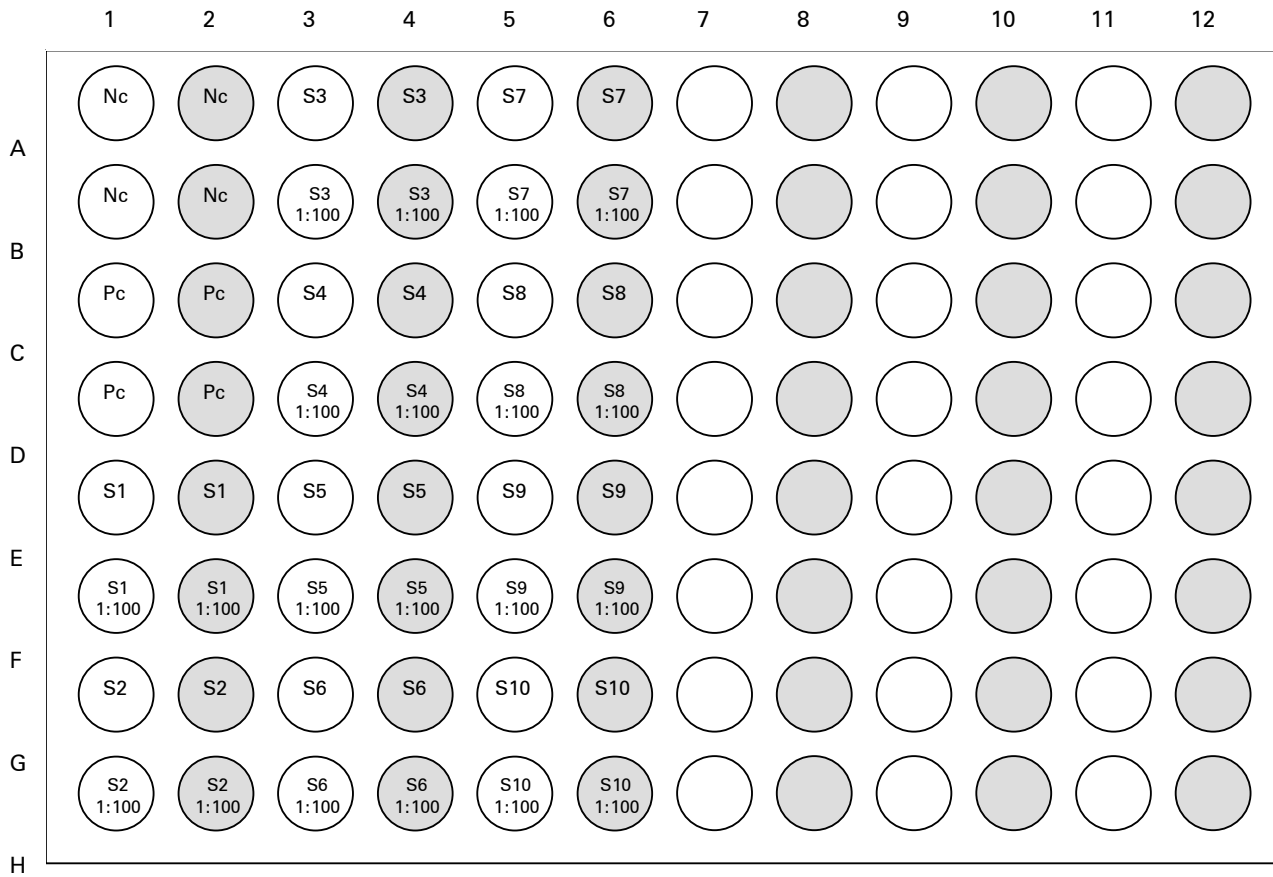
Screening procedure used: Confirmatory procedure used:

Procedure A	Procedure A
Procedure B	Procedure A or B
Procedure C	Procedure A, B or C
Procedure D	Procedure A, B or C

NOTE: Confirmation of a low HBsAg reactive specimen (absorbance value near the cut-off) may be difficult, because extra protein solution (confirmatory/control reagent) will decrease the sensitivity of the test. Thus it is recommended to use the most sensitive HBsAg assay conditions (Procedure A and the lower cut-off value; see page 13: CALCULATION OF THE CUT-OFF VALUE) when low HBsAg reactive specimens are confirmed.


STEP I

Pipetting scheme:



 = Confirmatory wells

Nc = Negative control

 = Confirmatory control wells

Pc = Positive control

S1 = Undiluted sample S1

S1= Diluted (1:100) sample S1 1:100

1. Pipette 25 µl of the confirmatory reagent (vial 1) into confirmatory wells (for example A1 - H1).
2. Pipette 25 µl of the control reagent (vial 2) into confirmatory control wells (for example A2 - H2).
3. Pipette 50 µl of conjugate into all Microstrip® wells.
4. Pipette 100 µl of the negative control into two confirmatory wells (eg. A1 and B1).
5. Pipette 100 µl of the negative control into two confirmatory control wells (eg. A2 and B2).
6. Pipette 100 µl of the positive control into two confirmatory wells (eg. C1 and D1).
7. Pipette 100 µl of the positive control into two confirmatory control wells (eg. C2 and D2).
8. Pipette 100 µl of undiluted specimens into one confirmatory well (eg. E1) and one confirmatory control well (eg. E2).
9. Pipette 100 µl of diluted (1:100) specimens into one confirmatory well (eg. F1) and one confirmatory control well (eg. F2).
10. Incubate according to the procedure chosen:

Procedure A: 120 minutes at +37°C with continuous shaking (900 rpm)

Procedure B: Static incubation 16-22 hours at room temperature

Procedure C: Static incubation 2-3 hours at +37°C

To proceed please refer to page 4, starting from Step I / Washing.

RESULTS

Quality Control Values

NEGATIVE CONTROL MEAN (NCx)

Individual negative control values should be less than or equal to 0.150 when the photometer is blanked against air, greater than or equal to 0.5 times NCx and less than or equal to 1.5 times NCx. If one of the values is outside the acceptable range, discard this value and recalculate the mean. If two of the values are outside the range, the test should be repeated.

POSITIVE CONTROL MEAN (PCx)

PCx should be greater than or equal to 0.400. The positive control should be reduced by at least 50 % with addition of confirmatory reagent. If not, the technique may be suspected and the run should be repeated.

Calculation of the Results

CALCULATION OF THE NEGATIVE CONTROL MEAN (NCx)

Determine the mean of the four negative control values (two neutralized and two non-neutralized).

Example:

Negative control Sample no.	Absorbance
1 (neutralized)	0.054
2 (neutralized)	0.048
3 (non-neutralized)	0.048
4 (non-neutralized)	0.050
Total	0.200

$$NCx = \frac{\text{Total absorbance}}{4} = \frac{0.200}{4} = 0.050$$

In the example, no negative control value is outside the quality control range and the NCx needs not to be revised.

CALCULATION OF THE POSITIVE CONTROL MEAN (PCx)

Determine the mean of the positive control (non-neutralized) values.

Example:

PC Sample no.	Absorbance
1 (non-neutralized)	1.080
2 (non-neutralized)	0.990
Total	2.070

$$PCx = \frac{\text{Total absorbance}}{2} = \frac{2.070}{2} = 1.035$$

In the example PCx is greater than 0.400, thus the technique is acceptable and data should be considered valid.

CALCULATION OF THE CUT-OFF VALUE (CO)

Determine the cut-off value by adding 0.05 or 0.1 to the negative control mean (NCx) for **Procedures A, B and C.**

$$CO = NCx + 0.05$$

Example: $CO = 0.050 + 0.05 = 0.100$

$$CO = NCx + 0.1$$

Example: $CO = 0.050 + 0.1 = 0.150$

CALCULATION OF THE PERCENTAGE OF REDUCTION FOR THE POSITIVE CONTROL AND FOR EACH SPECIMEN

Determine the percentage of reduction for positive control and for each specimen (undiluted and diluted) using the following equation:

$$\% \text{ Reduction} = \frac{A_{450} \text{ (non-neutralized)} - A_{450} \text{ (neutralized)}}{A_{450} \text{ (non-neutralized)} - A_{450} \text{ NCx}} \times 100$$

Example:

Sample	Absorbance
NCx	0.080
PCx (non-neutralized)	1.120
PCx (neutralized)	0.090
Undiluted specimen (non-neutralized)	2.000
Undiluted specimen (neutralized)	0.450
Diluted (1:100) specimen (non-neutralized)	1.870
Diluted (1:100) specimen (neutralized)	0.080

$$\begin{aligned} \text{Positive control: } & \frac{1.120 - 0.090}{1.120 - 0.080} = \frac{1.030}{1.040} \times 100 = 99 \% \text{ Reduction} \\ \\ \text{Specimen (undiluted): } & \frac{2.000 - 0.450}{2.000 - 0.080} = \frac{1.550}{1.920} \times 100 = 81 \% \text{ Reduction} \\ \\ \text{Specimen (diluted 1:100): } & \frac{1.870 - 0.080}{1.870 - 0.080} = \frac{1.790}{1.790} \times 100 = 100 \% \text{ Reduction} \end{aligned}$$

NOTE: If an absorbance value obtained is greater than or equal to 2.000, use the value 2.000 for calculation purposes.

Any specimen yielding less than 50 % neutralization and with an absorbance value for the non-neutralized diluted (1:100) specimen >2.000 should be diluted 1:250 or more and retested (see SPECIMEN DILUTION, on page 11).

Results

a) HBsAg negative:

A test sample is considered to be HBsAg negative if the absorbance value obtained (undiluted or diluted specimen) is not reduced at least 50 % by the addition of confirmatory reagent, and the absorbance value of the non-neutralized (undiluted or diluted) specimen is < 2.000.

NOTE: If the absorbance value of the diluted (1:100) non-neutralized specimen is > 2.000, and if the value is not reduced at least 50 % by the addition of the confirmatory reagent, the specimen has to be further diluted to reach an absorbance value of < 2.000; if the neutralization percentage for this dilution is still < 50 %, the specimen should be considered as HBsAg negative.

b) HBsAg positive:

A test sample is considered to be HBsAg positive if A_{450} (non-neutralized) for the specimen (undiluted or diluted) is greater than or equal to the HBsAg EIA Plus cut-off value, and the reactivity of the specimen (undiluted and/or diluted) is reduced 50 % or more by the addition of confirmatory reagent.

NOTE: A sample that is reduced more than 50 % but which generates a signal value below the cut-off level should be considered as suspect HBsAg positive. In case of a suspect HBsAg positive result it is recommended to confirm the results with a new serum sample.

Examples:

NCx = 0.080			
Specimen	A ₄₅₀ (non-neutralized)	A ₄₅₀ (neutralized)	% Reduction
1 (undil.)	2.000	0.450	81
1 (dil.)	1.870	0.080	100
2 (undil.)	2.000	2.000	0
2 (dil.)	2.000	2.000	0
3 (undil.)	2.000	2.000	0
3 (dil.)	2.000	1.800	10
4 (undil.)	0.320	0.310	4
4 (dil.)	0.090	0.090	0
5 (undil.)	1.050	1.350	0
5 (dil.)	2.000	1.900	5

Assay interpretation:

Specimen 1

Confirmed as HBsAg positive, because the reactivity of the specimen (undiluted and diluted) is reduced more than 50 %.

Specimen 2

Probably a high titer HBsAg positive specimen, because **it yields less than 50 % neutralization even as diluted and the absorbance values obtained with the non-neutralized specimen (undiluted and diluted) are > 2.000**. This specimen should be diluted 1:250 or more with sample diluent and tested again with the HBsAg Confirmatory Test.

Specimen 3

Probably a high titer HBsAg positive specimen, because **it yields less than 50 % neutralization even as diluted and the absorbance values obtained with the non-neutralized specimen (undiluted and diluted) are > 2.000**. This specimen should be diluted 1:250 or more with sample diluent and tested again with the HBsAg Confirmatory Test.

Specimen 4

Negative for HBsAg, because **the specimen yields less than 50 % neutralization and the absorbance values obtained with the non-neutralized specimen (undiluted and diluted) are < 2.000**.

Specimen 5

Probably a very high titer HBsAg positive specimen. The absorbance value obtained with **the undiluted non-neutralized** specimen is < 2.000. This may be caused by the hook effect, because the absorbance value for **the diluted non-neutralized** specimen is > 2.000. This specimen should be diluted 1:250 or more with sample diluent and tested again with the HBsAg Confirmatory

Test, because **the specimen yields less than 50 % neutralization and because the diluted non-neutralized specimen still yields an absorbance value > 2.000**.

Interpretation of the Results

A specimen which is repeatedly reactive with Ani Lab systems' HBsAg EIA Plus and is confirmed as positive with Ani Lab systems' HBsAg Confirmatory Test should be considered positive for HBsAg.

A specimen which is repeatedly reactive with Ani Lab systems' HBsAg EIA Plus but which is not confirmed as positive with Ani Lab systems' HBsAg Confirmatory Test should be considered negative for HBsAg.

As in any diagnostic enzyme immunoassay, there is the possibility that non-repeatable reactions may occur for the following reasons:

- Inadequate washing
- Contamination of reaction well with anti-HBs-HRP conjugate
- Contamination of substrate solution with conjugate or with oxidizing agents
- Cross-contamination of non-reactive specimens by HBsAg.

RELATED PRODUCTS AND ORDER INFORMATION:

Product number Product description

61 10 800	HBsAg EIA Plus (96 wells)
61 10 802	HBsAg EIA Plus (480 wells)
61 10 804	HBsAg EIA Plus (4800 wells)
61 06 070	HBsAg Confirmatory Test
61 10 370	Anti-HBc EIA
61 10 380	Anti-HBc IgM EIA
6200300N	Anti-HBs EIA
6200310N	HBe/anti-HBe EIA

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