

ANTIBODY TO HEPATITIS B SURFACE ANTIGEN (HUMAN)

GS HBsAg Confirmatory Assay 3.0

For Confirmatory Neutralization of
GS HBsAg EIA 3.0 Reactive Specimens

For *In Vitro* Diagnostic Use 32594

NAME AND INTENDED USE

The GS HBsAg Confirmatory Assay 3.0 is a qualitative assay intended for the confirmation of HBsAg reactive specimens detected in the GS HBsAg EIA 3.0.

SUMMARY AND EXPLANATION OF THE TEST

Hepatitis B virus (HBV) is a major public health problem worldwide, with significant transmission of the virus occurring through the use of contaminated donor blood and plasma. Because the presence of circulating Hepatitis B Surface Antigen (HBsAg) closely follows the course of infection, screening for HBsAg is used to detect potentially infectious blood and plasma.¹ Enzyme immunoassays to detect HBsAg have replaced relatively insensitive gel diffusion methods, and have been reported to have equivalent sensitivity to radioimmunoassay methods.² The application of monoclonal antibodies for the detection of HBsAg has previously been reported.^{3,4}

The GS HBsAg Confirmatory Assay 3.0 is an HBsAg neutralization procedure using anti-HBs (Human) to confirm the presence of HBsAg in specimens found to be repeatedly reactive by the GS HBsAg EIA 3.0. A repeatedly reactive specimen should be confirmed by a licensed neutralization procedure utilizing human anti-HBs (HBsAg Confirmatory Assay). Only those specimens in which the HBsAg can be neutralized by the confirmatory test procedure may be designated as positive for HBsAg.

BIOLOGICAL PRINCIPLES OF THE PROCEDURE

The repeatedly reactive specimen is incubated with HBsAg Confirmatory Reagent [Antibody to Hepatitis B Surface Antigen (Human)]. If HBsAg is present in the specimen, it will be neutralized by the HBsAg Confirmatory Reagent. The treated specimen is re-assayed using the GS HBsAg EIA 3.0. The neutralized HBsAg is prevented from binding to the HBsAg antibody-coated microwells, which results in a reduction of signal.

A non-neutralized control of the specimen [treated with HBsAg Negative Control (Human) in place of HBsAg Confirmatory Reagent] is tested in parallel to the neutralized specimen for comparison of signal.

GS HBsAg EIA 3.0 repeatedly reactive specimens are confirmed positive by the GS HBsAg Confirmatory Assay 3.0 if the reduction in signal of the neutralized specimen is greater

than or equal to 50% of the non-neutralized sample and the non-neutralized specimen signal is greater than or equal to the assay cutoff.

REAGENTS

GS HBsAg Confirmatory Assay 3.0 Product No: 32594, Number of Tests: 25		
Component	Contents	Preparation
1. Antibody to Hepatitis B Surface Antigen (Human) (HBsAg Confirmatory Reagent) 1 vial (1.0 mL)	<ul style="list-style-type: none"> Antibody to HBsAg (Human) 0.005% Gentamicin Sulfate 0.03% Proclin® 5000 	Ready to use as supplied.
2. HBsAg Negative Control (Human) 1 bottle (12 mL)	<ul style="list-style-type: none"> Normal Human Serum/Plasma Non-reactive for HBsAg and Anti-HBsAg 0.005% Gentamicin Sulfate 0.03% Proclin® 5000 	Ready to use as supplied.

Store the kit at 2-8°C. Bring all reagents to room temperature (15-30°C) before use. Return to 2-8°C immediately after use.

WARNINGS FOR USERS

For *In Vitro* Diagnostic Use

- The GS HBsAg Confirmatory Assay 3.0 and GS HBsAg EIA 3.0 contain human blood components. No known test method can offer complete assurance that products derived from human blood will not transmit infection. Therefore, all human blood derivatives should be handled as though they contain an infectious agent. Handle these reagents and human specimens using the precautions recommended for bloodborne pathogens, as defined by OSHA regulations.
- Do not pipette by mouth.
- Do not smoke, drink, or eat in areas where specimens or kit reagents are being handled.
- Wear protective clothing and disposable gloves while handling the kit reagents. Wash hands thoroughly after performing the test.
- The Stopping Solution, supplied with the GS HBsAg EIA 3.0, is a strong acid. Wipe up spills immediately. Flush the area of the spill with water. If the Stopping Solution contacts the skin or eyes, flush with plenty of water and seek medical attention.
- BIOLOGICAL SPILLS:** Spills not containing acid should be wiped thoroughly with an effective disinfectant. Disinfectants that are known to inactivate the virus include (but are not limited to) a solution of 10% bleach (0.5% solution of sodium hypochlorite), 70% ethanol, or 0.5% Wescodyne™.⁵⁻⁷
Spills containing acid should be wiped dry. The area of the spill should be wiped with one of the chemical disinfectants. Materials used to wipe up spills should be disposed of as biohazardous waste.
NOTE: DO NOT PLACE SOLUTIONS CONTAINING BLEACH IN THE AUTOCLAVE.
- Dispose of all specimens and materials used to perform the test as though they contain an infectious agent. Disposal should comply with all applicable waste disposal requirements.

PRECAUTIONS FOR USERS

- Do not use the kit beyond the stated expiration date.
- The cutoff calculations for the GS HBsAg EIA 3.0 and GS HBsAg Confirmatory Assay 3.0 are not the same. Do not use the cutoff calculations in the GS HBsAg EIA 3.0 package insert for confirmatory testing.**
- Exercise care when opening vials and removing aliquots to avoid microbial contamination of the reagents.
- Bring all reagents except the HBsAg EIA 3.0 Conjugate Concentrate to room temperature before use.
- For the manual pipetting of controls and specimens, use individual pipette tips to eliminate carryover of samples.
- Handle negative and positive controls in the same manner as patient specimens.

7. If a specimen or reagent is inadvertently not added to a well, the assay results will read negative.
8. Inadequate adherence to package insert instructions may result in erroneous results.
9. Use only adequately calibrated equipment with this assay.
10. Use of dedicated equipment is recommended if equipment performance validations have not precluded the possibility of cross-contamination.
11. The GS HBsAg Confirmatory Assay 3.0 performance is highly dependent upon incubation times and temperatures. Temperatures outside of the validated ranges may result in invalid assays. Incubation temperatures should be carefully monitored using calibrated thermometers, or equivalent.
12. Components of this kit meet FDA potency requirements.

SPECIMEN COLLECTION, PREPARATION, AND STORAGE

The specimens to be tested by the GS HBsAg Confirmatory Assay 3.0 are those found to be repeatedly reactive by the GS HBsAg EIA 3.0 screening assay.

Serum, plasma, or cadaveric serum specimens may be used. The following anticoagulants in glass tubes have all been evaluated and found to be acceptable: EDTA, sodium heparin, sodium citrate, CPDA-1, and ACD. In addition, plastic tubes with serum, serum separators, and the following anticoagulants have also been evaluated and found to be acceptable: EDTA, lithium heparin, and sodium citrate. Samples which are collected into anticoagulant tubes should be filled as labeling indicates to avoid improper dilution. Specimens with observable particulate matter should be clarified by centrifugation prior to testing. No clinically significant effect has been detected in assay results of serum or plasma samples with increased levels of protein, lipids, bilirubin, or hemolysis, or after heat inactivation of patient samples. Cadaveric serum samples with increased levels of hemolysis have been tested, and no clinically significant effect has been detected in assay results. Note: Cadaveric serum samples with increased levels of protein, lipids, bilirubin, or microbiological contaminants have not been available to evaluate with this assay.

Serum, plasma, or cadaveric serum specimens may be stored at 2-8°C for up to seven days. Samples should not be used if they have incurred more than 5 freeze/thaw cycles. Mix samples thoroughly after thawing. NOTE: Cadaveric specimens that are weakly reactive may become nonreactive after freeze/thaw cycles.

If specimens are shipped, they should be packed in compliance with Federal Regulations covering the transportation of etiologic agents. Studies have demonstrated that specimens may be shipped refrigerated (2-8°C) or at ambient temperatures for up to 7 days. For shipments that are in transit for more than 7 days, specimens should be kept frozen (-20°C or lower). Refrigerate samples at 2-8°C at receipt, or freeze for longer storage.

This kit is not intended for use with specimens other than serum, plasma, and cadaveric serum specimens. This kit is not intended for use on saliva/oral fluids or urine samples.

GS HBsAg CONFIRMATORY ASSAY 3.0 PROCEDURE Materials Provided

See Reagents Section on page 2.

Materials Required but not Provided

1. GS HBsAg EIA 3.0.
2. Plastic test tubes.
3. Precision pipettes to deliver volumes from 10 µL to 200 µL, 1 mL, 5 mL, and 10 mL (accurate within ± 10%). A multichannel pipettor capable of delivering 100 µL or 200 µL is optional.
4. Pipette tips.
5. Dry-heat static or shaker incubator capable of maintaining 37±1°C. If a shaker incubator is used, it should have the following specifications:

Amplitude: 0.75 to 3.00 mm
Frequency: 500 to 2300 R.P.M.

6. Microwell plate or strip washer qualified for use with this assay. The washer must be capable of dispensing at least 400 µL per well, cycling 5 times, and soaking for 30-60 seconds between each wash.
7. Microwell plate or strip reader qualified for use with this assay. The spectrophotometer should have the following specifications at wavelength 450 nm:
Bandwidth: 10 nm HBW (Half Band Width) or equivalent
Absorbance Range: 0 to 2 AU (Absorbance Units)
Repeatability: ± (0.5% + 0.005) AU
Linearity or Accuracy: 1% from 0 to 2.0 AU
The instrument should contain a reference filter for reading at 615 to 630 nm. An instrument without a reference filter can be used; however, areas in the bottoms of the wells that are opaque, scratched or irregular may cause absorbance readings that are falsely elevated.
8. Household bleach (5% to 8% sodium hypochlorite) which may be diluted to a minimum concentration of 10% bleach (or 0.5% sodium hypochlorite). Alternative disinfectants include: 70% ethanol or 0.5% Wescodyne™ (West Chemical Products, Inc.).
9. Paper towels or absorbent pads for blotting.
10. Labeled null strips, for testing partial plates.
11. Clean, polypropylene containers for preparation of TMB and Conjugate Working Solutions, 15 or 50 mL.
12. Deionized water or distilled water. Clinical laboratory reagent water Type I or Type II is acceptable.
13. Gloves.
14. Laboratory timer.
15. EIA reagent reservoirs (optional)

Preliminary Statements

1. The expected run time for this procedure is 4 hours. Each run of this assay must proceed to completion without interruption after it has been started.
2. Positive and Negative Controls must be run on each plate. The cutoff for patient samples is determined by the controls on each individual plate.
3. The number of controls to be included in each run of this assay are four Positive Controls (two neutralized and two non-neutralized), four Low Positive Controls (two neutralized and two non-neutralized) and three Negative Controls (non-neutralized).
4. Do not splash controls, specimens, or reagents between microwells of the plate.
5. Cover plates for each incubation step using plate covers provided or other appropriate means to minimize evaporation.
6. Avoid exposure of the plates to light during the final incubation step (following the addition of the Working TMB Solution).
7. Adhere to the recommended time constraints for the use of the Working TMB Solution (8 hours), Working Conjugate Solution (8 hours) and Wash Solution (4 weeks).
8. Avoid the formation of air bubbles in each microwell.

EIA Procedure

There are two procedures for the confirmation of HBsAg in human serum or plasma, procedures A and B. **For the confirmation of HBsAg in cadaveric serum specimens, only procedure A can be used.** The two procedures for the confirmation of HBsAg are described below:

Procedure	Specimen incubation	Conjugate incubation	Color development
A	static incubation, dry heat, 36-38°C, 60 minutes.	static incubation, dry heat, 36-38°C, 60 minutes.	15 to 30°C; 30 min. in the dark
B	shaker incubation, 36-38°C, 60 min.	shaker incubation, 36-38°C, 60 min.	15 to 30°C; 30 min. in the dark

Note: Specimens found to be repeatedly reactive by the GS HBsAg EIA 3.0 using static incubations (Procedure A) should be confirmed with GS HBsAg Confirmatory Assay 3.0 using static incubations (Procedure A). Specimens found to be repeatedly reactive by the GS HBsAg EIA 3.0 using

shaker incubations (Procedure B) should be confirmed with GS HBsAg Confirmatory Assay 3.0 using shaker incubations (Procedure B).

1. Perform equipment maintenance and calibration, where necessary, as required by the manufacturer.
2. **Bring all of the reagents from the GS HBsAg EIA 3.0 and GS HBsAg Confirmatory Assay 3.0, except the HBsAg Conjugate Concentrate, to room temperature before beginning the assay procedure.**
3. Prepare Working Wash Solution, Working Conjugate Solution, and Working TMB Solution as described in the GS HBsAg EIA 3.0 Reagent Preparation and Storage section of the package insert. Mix gently, by inversion. Mix again just before use.
4. Remove strips not needed for the assay and replace them with labeled Null Strips, if necessary.
5. If sample identity is not maintained by an automatic procedure, identify the individual wells for each specimen or control on a data sheet.
6. **Neutralization Procedure**
 - a. For each specimen, label one plastic test tube "A" (neutralized) and one plastic test tube "B" (non-neutralized control). Pipette 240 μ L of repeatedly reactive specimen into each tube.
 - b. For HBsAg Positive Control (Human), label one plastic test tube "A" (neutralized) and one plastic test tube "B" (non-neutralized). Pipette 240 μ L of HBsAg Positive Control (Human) from the GS HBsAg EIA 3.0 kit into each tube.
 - c. For HBsAg Low Positive Control (Human), label one plastic test tube "A" (neutralized) and one plastic test tube "B" (non-neutralized). Pipette 240 μ L of HBsAg Low Positive Control (Human) from the GS HBsAg EIA 3.0 kit into each tube.
 - d. For HBsAg Negative Control (Human), label one plastic test tube "B" (non-neutralized). Pipette 360 μ L of HBsAg Negative Control (Human) from the GS HBsAg EIA 3.0 kit into the tube.
 - e. To each tube labeled "A" (neutralized) add 30 μ L of HBsAg Confirmatory Reagent.
 - f. To each tube labeled "B" (non-neutralized), **except for the Negative Control, add 30 μ L of HBsAg Negative Control (Human) from the GS HBsAg Confirmatory Assay 3.0 kit. To the Negative Control tube labeled "B" add 45 μ L of HBsAg Negative Control (Human) from the GS HBsAg Confirmatory Assay 3.0 kit.**
 - g. Mix each tube by gentle vortex mixing or tapping. Avoid excessive foaming.
 - h. Incubate the tubes for 15-30 minutes at room temperature to allow the neutralization reaction to proceed.
7. **Add 100 μ L of the controls or specimens to the wells of the microwell plate.** Four Positive Controls (two "A" and two "B"), four Low Positive Controls (two "A" and two "B"), and three Negative Controls should be assayed on each plate or partial plate of specimens. Four wells should be assayed for each specimen, two neutralized ("A") and two non-neutralized ("B").
8. Cover the microwell plate with a plate cover or use other means to minimize evaporation.

Procedure A: Incubate the plate for 60 to 65 minutes at 37 \pm 1 $^{\circ}$ C using a dry-heat static incubator.

Procedure B: Incubate the plate for 60 to 65 minutes at 37 \pm 1 $^{\circ}$ C using a shaker incubator.
9. At the end of the incubation period, carefully remove the plate cover, and aspirate the fluid from each well into a biohazard container. **Wash the microwell plate or strip a minimum of five times with the Wash Solution (at least 400 μ L/well/wash). Soak for 30 to 60 seconds between each wash.** Aspirate the Wash Solution after each wash. After the last wash, if excess liquid remains, blot the inverted plate on clean, absorbent paper towels. *Note: Grasp the plate holder firmly at the center of the long sides before inverting to blot.*

10. **Add 100 μ L of Working Conjugate Solution to each well** containing a specimen or control.
11. Cover the microwell plate with a plate cover or use other means to minimize evaporation.

Procedure A: Incubate the plate for 60 to 65 minutes at 37 \pm 1 $^{\circ}$ C using a dry-heat static incubator.

Procedure B: Incubate the plate for 60 to 65 minutes at 37 \pm 1 $^{\circ}$ C using a shaker incubator.
12. At the end of the incubation period, carefully remove the plate cover and aspirate the fluid from each well into a biohazard container. **Wash the microwell plate or strip a minimum of five times with the Wash Solution (at least 400 μ L/well/wash). Soak for 30 to 60 seconds between washes.** Aspirate the Wash Solution after each wash. After the fifth wash, if excess liquid remains, blot the inverted plate on clean, absorbent paper towels. *Note: Grasp plate firmly at the center of the long sides before inverting to blot.*
13. **Add 100 μ L of the Working TMB Solution to each well.** Cover the microwell plate with a fresh plate cover or use other means to minimize evaporation, and **incubate plates in the dark for 30 to 33 minutes at room temperature.** (For example, cover the plates with black plastic or place them in a drawer.)
14. Carefully remove the plate cover, and **add 100 μ L of EIA Stopping Solution to each well** to terminate the reaction. **Tap plate gently or use other means to assure complete mixing. Complete mixing is required for acceptable results.**
15. **Read absorbance within 30 minutes** after adding the EIA Stopping Solution, using the 450 nm filter with 615 nm to 630 nm as the reference. Ensure that all strips have been pressed firmly into place before reading.

Decontamination

Dispose of all specimens and materials used to perform the test as though they contain an infectious agent. Disposal should comply with all applicable waste disposal requirements.

QUALITY CONTROL

Determine the mean absorbance for the Negative, Positive, and Low Positive Controls (both neutralized and non-neutralized), by dividing the summation of their absorbance values by the number of acceptable controls. One Negative Control may be discarded if it is outside of the acceptable validation range. No Positive Controls or Low Positive Controls may be discarded. For the procedure to calculate mean absorbance values, follow the criteria in the Quality Control section of the GS HBsAg EIA 3.0 package insert.

Confirmation of the presence of HBsAg is determined by comparing the mean absorbance value (X_{abs}) of the neutralized samples to the mean absorbance value (X_{non}) of the non-neutralized samples. This comparison is expressed as % reduction. The % reduction for all controls and samples is determined by applying the following equation:

$$\% \text{ reduction} = 100 \times \frac{(X_{non} \text{ non-neutralized sample} - X_{abs} \text{ neutralized sample})}{(X_{non} \text{ non-neutralized sample} - X_{abs} \text{ Negative Control})}$$

Example:

Sample	Mean Absorbance
Negative Control, non-neutralized	0.025
Positive Control, non-neutralized	1.720
Positive Control, neutralized	0.016
% reduction of HBsAg Positive Control (PCX) =	
$100 \times \frac{1.720 - 0.016}{1.720 - 0.025} = \frac{1.704}{1.695} = 100.5\% \text{ reduction}$	
Low Positive Control, non-neutralized	0.381
Low Positive Control, neutralized	0.024
% reduction of HBsAg Low Positive Control (LPCX) =	
$100 \times \frac{0.381 - 0.024}{0.381 - 0.025} = \frac{0.357}{0.356} = 100.3\% \text{ reduction}$	

A run is valid if the following criteria are met:

- The absorbance values of the Negative Controls are greater than 0.000 AU and less than or equal to 0.100 AU. One Negative Control value may be discarded. If two or more Negative Controls are out of limit, the run must be repeated.
- The mean absorbance value of the non-neutralized Positive Controls (PCX) must be greater than or equal to 1.000 AU and the individual absorbance values must be within range of 0.65 to 1.35 times the PCX. No Positive Control values may be discarded.
- The mean absorbance value of the non-neutralized Low Positive Controls (LPCX) must be positive (greater than or equal to the assay cutoff). No Low Positive Control values may be discarded.
- The % reduction of each neutralized HBsAg Positive Control and Low Positive Control is $\geq 50\%$.

INTERPRETATION OF RESULTS

Note: If the absorbance value of patient samples is greater than the upper linearity limits of the reader, use the reader upper cutoff as the absorbance value for the following calculations.

1. Determine the X_{abs} for the neutralized ("A") and non-neutralized ("B") specimens.
2. Calculate the cutoff value by adding 0.035 to the Negative Control mean.
3. Determine the % reduction of each specimen X_{abs} using the equation in the Quality Control section.

An example of values obtained from an assay run and the interpretation are as follows:

Sample	Absorbance values	Mean Absorbance
Negative Control, non-neutralized	0.031	0.025
	0.020	
	0.024	
Positive Control, non-neutralized	1.683	1.720
	1.757	
Positive Control, neutralized	0.013	0.016
Low Positive Control, non-neutralized	0.360	0.381
	0.402	
Low Positive Control, neutralized	0.020	0.024
Specimen, non-neutralized	0.861	0.858
	0.855	
Specimen, neutralized	0.025	0.021
Specimen cutoff	$= 0.025 + 0.035 = 0.060$	
% reduction of HBsAg PCX = $100 \times \frac{1.720 - 0.016}{1.720 - 0.025}$	$= \frac{1.704}{1.695} = 100.5\%$ reduction	
% reduction of HBsAg LPCX = $100 \times \frac{0.381 - 0.024}{0.381 - 0.025}$	$= \frac{0.357}{0.356} = 100.3\%$ reduction	
% reduction of specimen $X_{abs} = 100 \times \frac{0.858 - 0.021}{0.858 - 0.025}$	$= \frac{0.837}{0.833} = 100.5\%$ reduction	

Note: In some instances, high titer HBsAg specimens will not show a $\geq 50\%$ reduction in signal by the addition of HBsAg Confirmatory Reagent. Therefore, highly reactive specimens (absorbance ≥ 2.000) should be diluted, e.g. 1:10 or 1:100, in HBsAg Negative Control (human) and assayed once again by the GS HBsAg Confirmatory Assay 3.0. If the % reduction is still not $\geq 50\%$ for the diluted specimen and the diluted specimen is still highly reactive, redilute the first dilution 1:100 or greater in HBsAg Negative Control (Human), and assay again by the GS HBsAg Confirmatory Assay 3.0.

A specimen is considered to be positive for HBsAg if the following criteria are met:

- The specimen is repeatedly reactive by the GS HBsAg EIA 3.0.
- The absorbance value of the non-neutralized specimen is greater than or equal to the calculated cutoff value of the GS HBsAg Confirmatory Assay 3.0.
- The % reduction of the specimen X_{abs} is $\geq 50\%$.

LIMITATIONS OF THE PROCEDURE

1. The GS HBsAg EIA 3.0 Procedure and GS HBsAg Confirmatory Assay 3.0 Procedure package insert recommendations must be followed when testing serum, plasma, or cadaveric serum specimens for the presence of HBsAg. The user of this kit is advised to read the package insert carefully prior to conducting the test. In

particular, the test procedure must be carefully followed for specimen and reagent pipetting, plate washing and timing of the incubation steps.

2. False negative results can occur if the quantity of marker present in the sample is too low for the detection limits of the assay, or if the marker which is detected is not present during the stage of disease in which a sample is collected.
3. Failure to add specimen or reagent as instructed in the procedure could result in a falsely negative test. Repeat testing should be considered where there is clinical suspicion of infection or procedural error.
4. An absorbance value of less than 0.000 AU may indicate a procedural or instrument error which should be evaluated. That result is invalid and that specimen must be re-run.
5. Factors that can affect the validity of results include failure to add the specimen or reagent to the well, inadequate washing of microplate wells, failure to follow stated incubation times and temperatures, addition of wrong reagents to wells, the presence of metals, or splashing of bleach into wells.

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