Caution - Federal law restricts this device to sale by or on the order of a physician.

1. INTENDED USE

ETI-MAK-2 PLUS is an *in vitro* enzyme immunoassay (EIA) intended for use in the qualitative determination of hepatitis B surface antigen (HBsAg) in human serum or plasma (EDTA, citrate or heparin). The ETI-MAK-2 PLUS is intended for manual use and with the Biochem Immunosystems Labotech/ETI-LAB automated instrument.

The detection of HBsAg is indicative of a laboratory diagnosis for hepatitis B virus (HBV) infection, either acute or chronic. Further HBV serological marker testing is required to define the specific disease state. The ETI-MAK-2 PLUS assay's performance has not been established for the monitoring of HBV disease or therapy.

WARNING - This assay has not been FDA-approved for the screening of blood or plasma donors.

WARNING - Assay performance characteristics have not been adequately established for prenatal screening. All results must be confirmed with additional HBV marker testing to include confirmatory neutralization testing of all positive results.

Assay performance characteristics have not been established when the ETI-MAK-2 PLUS HBsAg assay is used in conjunction with other manufacturers' assays for specific HBV serological markers. Users are responsible for establishing their own performance characteristics.

The performance characteristics of this assay have not been established for newborn testing.

Caution - Performance characteristics for ETI-MAK-2 PLUS were largely determined using archival specimens which may not be representative of test results obtained from fresh specimens. Laboratories are advised that they should monitor patient results using other appropriate HBV serological markers or retest questionable specimens with another legally-marketed HBsAg assay.

2. SUMMARY AND EXPLANATION OF THE TEST

Hepatitis is an inflammatory disease of the liver that can severely damage the organ. The disease can result from non-infectious causes—such as biliary obstruction, biliary cirrhosis, Wilson's disease, drug toxicity, and drug hypersensitivity reactions—or from infectious viral and bacterial agents (1). Viral hepatitis is commonly caused by one of several viruses: hepatitis A virus (HAV), hepatitis B virus (HBV), hepatitis C virus (HCV), or hepatitis D (delta) virus (HDV) in conjunction with HBV (1, 2), hepatitis E virus (HEV), and other as yet uncharacterized or partially characterized hepatitis viruses (non-A-E). Other viruses, including yellow fever virus, human cytomegalovirus, Epstein-Barr virus, rubella virus, herpes simplex virus, varicella-zoster virus, and some enteroviruses, can cause forms of hepatitis (1, 2).

Hepatitis B, also known as serum hepatitis, is endemic throughout the world (3-5). The infection is spread primarily through percutaneous contact with infected blood or blood products, e.g., sharing of needles by drug addicts or transfusion of blood products that have not been screened for HBV (1, 3, 6, 7). The virus is also found in virtually every type of human body fluid and has been known to be spread through oral and genital contact (1, 3, 6, 7). HBV can be transmitted from mother to child

during delivery through contact with blood and vaginal secretions; it is not commonly transmitted transplacentally (6).

The incubation period for hepatitis B averages 90 days (range 40-180 days). Common symptoms include malaise, fever, gastroenteritis, and jaundice (icterus) (8). HBV infection can lead to a) icteric hepatitis, b) subclinical anicteric hepatitis, c) fulminant hepatitis, or d) chronic active or persistent hepatitis (1, 3). Over 90% of adult patients with hepatitis B completely recover from acute illness, approximately 1% die of fulminant hepatitis, and approximately 6 to 10% become chronic active or persistent carriers (1-3).

The complete hepatitis B virus, also called the Dane particle, is composed of an outer surface or envelope that carries the hepatitis B surface antigen (HBsAg) (9, 10). The envelope surrounds an inner core that contains the hepatitis B core antigen (HBcAg) (11-13). Inside the core is the HBV deoxyribonucleic acid (DNA) genome. Another antigen, the hepatitis B *e* antigen (HBeAg), is a viral core protein found in the bloodstream during active replication of HBV (14).

Because HBV is very difficult to isolate in cell culture, hepatitis B diagnosis has been based on detection of serologic markers. Early methods used to detect serologic markers were immunodiffusion and counterimmunoelectrophoresis (3). Methods commonly used now include hemagglutination, immune adherence, latex agglutination, radioimmunoassay (RIA), and enzyme immunoassay (EIA) (1, 3). The EIA and RIA methods are the most widely utilized because of their high analytical sensitivity, analytical specificity, and ease of use.

When determining the stage of disease caused by HBV the HBV serologic markers commonly tested for are HBsAg, antibody to HBsAg (anti-HBs), total antibody to HBcAg (total anti-HBc), immunoglobulin M antibody to HBcAg (IgM anti-HBc), HBeAg, and antibody to HBeAg (anti-HBe). Testing for these markers helps determine the presence of past or ongoing HBV infection, the acute or chronic stage of the disease, response to therapy, and/or the immune status of the patient (1, 15).

HBsAg is the viral component usually found in the highest concentration in the serum of HBV-infected patients (1, 6). It is a heterogeneous antigen. The principal determinant is called *a* and is common to all types of HBsAg. Other major determinants of the antigen are *d/y* and *w/r*. These determinant pairs are mutually exclusive, i.e., only the combinations *adw*, *adr*, *ayw*, and *ayr* are possible (10, 16).

Presence of HBsAg in serum may indicate a) acute HBV infection, b) chronic HBV infection, or c) asymptomatic carrier state (15, 17). The significance of HBsAg in serum is determined by evaluating it in relationship to the presence or absence of the other HBV markers and the clinical presentation and history of the patient.

3. PRINCIPLE OF THE PROCEDURE

ETI-MAK-2 PLUS uses monoclonal antibodies to hepatitis B surface antigen (HBsAg) as the basis for this enzyme immunoassay. The assay is a direct, non-competitive test based on the use of polystyrene microwells coated with mouse monoclonal antibodies to HBsAg (directed to the "a" determinant of HBsAg). An enzyme tracer containing horseradish peroxidase-labeled sheep antibodies to HBsAg detects any captured HBsAg from the patient's specimen.

In the assay procedure, patient specimens and controls are incubated with incubation buffer in anti-HBs-coated microwells. If HBsAg is present in a specimen or control, it binds to the antibodies. Excess sample is removed by a wash step, and the enzyme tracer is then added to the microwells and allowed to incubate. The enzyme tracer binds to any antigen-antibody complexes present in the microwells. Excess enzyme tracer is removed by a wash step, and a chromogen/substrate solution is added to the microwells and allowed to incubate. If a sample contains HBsAg, the bound enzyme (horseradish peroxidase) chemically reduces the substrate peroxide, which concurrently oxidizes the chromogen tetramethylbenzidine (TMB) to a blue color (650 nm). The blue color turns to yellow (450

nm) after addition of the stop solution. If a sample does not contain HBsAg, the microwell will be colorless after the chromogen/substrate solution is added and will remain colorless after the stop solution is added. Color intensity, which is measured spectrophotometrically, is indicative of the concentration of HBsAg. Absorbance value readings for patient specimens are compared to a cutoff value determined from the mean of the calibrators.

4. REAGENTS AND OTHER MATERIALS PROVIDED

Catalog Number	Product Description	Quantity/ Volume
P001932	**ETI-MAK-2 PLUS**	192 tests
	Coated Strips Microwells coated with mouse monoclonal antibodies to HBsAg (IgG1-k class, directed to the "a" determinant of HBsAg).	Twenty-four 8-well strips (contained in 2 pouches)
	Enzyme Tracer Horseradish peroxidase-labeled sheep IgG antibodies to HBsAg, buffer, protein stabilizers. Preservative: 0.2% ProClin 300.	0.7 mL
	Tracer Diluent Buffer, human serum/plasma, protein stabilizers. Preservative: 0.2% ProClin 300.	Two 14.7-mL vials
	Calibrator (Human) Human serum/plasma non-reactive for HBsAg. Preservative: 0.2% ProClin 300.	3.3 mL
	Negative Control (Human) Human serum/plasma non-reactive for HBsAg. Preservative: 0.2% ProClin 300.	3.3 mL
	Positive Control (Human) Human serum/plasma reactive for HBsAg (subtypes *ad* and *ay*), protein stabilizers. Preservative: 0.2% ProClin 300.	2.5 mL
	Incubation Buffer Buffer, protein stabilizers, an inert blue dye. Preservative: 0.2% ProClin 300.	16 mL
	Wash Buffer (concentrate)* Buffer, detergents, preservatives.	Two 40-mL vials
	Chromogen/Substrate* Tetramethylbenzidine/hydrogen peroxide system.	Two 16-mL vials
	Stop Solution* 1N sulfuric acid. Caution: corrosive.	30 mL
	Strip Sealers	48
	Plate Sealers	4
	Pouch Sealer	1

* *All lots of wash buffer concentrate, chromogen/substrate and stop solution are interchangeable between assay kits.*

5. WARNINGS AND PRECAUTIONS

- For *in vitro* diagnostic use only.
- The human blood source material used to prepare this product (e.g. Tracer Diluent, Calibrator and controls) has been tested and found non-reactive for antibodies to HCV, HIV-1 and HIV-2 by FDA-approved methods. Because no test method can offer complete assurance that laboratory specimens do not contain HIV, hepatitis B virus, or other infectious agents, specimens should be handled at the BSL 2 as recommended for any potentially infectious human serum or blood specimen in the CDC-NIH manual, Biosafety in Microbiological and Biomedical Laboratories, 4th Edition, May 1999 and NCCLS Approved Guideline M29-A, Protection of Laboratory Workers from Instrument Biohazards and Infectious Disease Transmitted by Blood, Body Fluids, and Tissue. (18, 19, 20)
- All specimens, reagents, and controls should be handled as if capable of transmitting disease. Follow standard precautions for handling infectious agents during all procedures:
  - Do not pipette by mouth.
  - Do not eat, drink, smoke, or apply cosmetics in areas where specimens are handled.
  - Wear protective clothing such as lab coats, protective glasses, and disposable gloves when handling specimens and assay reagents. Wash hands thoroughly afterwards.
  - Perform all work with infectious materials in a designated area.
- Dispose of all specimens and used assay materials as if capable of transmitting disease:
  - Decontaminate liquid wastes, including those containing neutralized acid, either:

    (a) by autoclaving for 60 minutes at 121°C; or

    (b) by treating with a 1:10 or 1:100 dilution of household bleach (sodium hypochlorite concentration approximately 5%). The wastes should remain in contact with the sodium hypochlorite solution for 30 minutes for effective decontamination, after which they can be disposed of in the sink (18, 20). Do not autoclave solutions containing sodium hypochlorite.
  - Autoclave non-ignitable solids for 60 minutes at 121°C.
  - Incinerate disposable ignitable materials.
- Performing the assay outside the time and temperature ranges provided may produce invalid results. Assays not falling within the established time and temperature ranges must be repeated.
- Use only dispensing equipment that has been calibrated to deliver accurate volumes, per the laboratory's standard procedures.
- **WARNING - Chromogen/substrate and the stop solution contain ingredients that can irritate skin and cause eye damage. Handle them with care. Avoid getting them in eyes or on skin or clothing. In case of contact with skin or eyes, immediately flush the affected area with water for 15 minutes. For eyes, obtain medical attention.**

  Reagents containing ProClin 300 may cause allergic reactions. Avoid prolonged contact with skin. Wash thoroughly after handling.

6. REAGENT PREPARATION

- Bring reagents to room temperature (20-25°C).

- The coated strips, calibrator, negative and positive controls, incubation buffer, chromogen/substrate and stop solution are provided ready to use.
  ***Note** - Use clean, plastic containers or acid-washed glassware for preparing the following solutions. A clean, dedicated dispenser is recommended for the working enzyme tracer to avoid contamination.*
- **Working enzyme tracer.** Bring reagents to room temperature. To prepare the working enzyme tracer, dilute the enzyme tracer 1:50 with tracer diluent (see chart below). After dilution, the working enzyme tracer can be used for one week if stored at 2-8°C.
  ***Caution** - Verify that the total volume prepared is sufficient for the number of tests included in the run. Use a clean container for each dilution and label the container with the reagent name, lot number of kit, lot number of reagent, plus the date of preparation and date of expiration of the working enzyme tracer.*

Number of Strips	Enzyme Tracer (µL)	Tracer Diluent (µL)	Total Volume (mL)
2	48	2352	2.4
4	80	3920	4.0
6	112	5488	5.6
8	144	7056	7.2
10	176	8624	8.8
12	208	10192	10.4

***Note** - Sufficient reagents are provided to allow for six runs per kit.*

- **Wash solution.** To prepare the working wash buffer, dilute the wash buffer concentrate (40 mL) to 1000 mL (1.0 L) with distilled or deionized water. If crystallization has occurred at 2-8°C, warm the wash buffer concentrate to 37°C and mix well before diluting. Water used for wash buffer dilution should be stored in a clean, non-metallic container to prevent contamination with peroxidase-inactivating substances. Record on the storage vial the expiration date and date of preparation of the working wash buffer. The working wash buffer can be stored for one week at 2-8°C.
  Smaller volume users may prepare less than 1 L of working wash buffer at a time if desired. If diluting only a portion of the wash buffer concentrate, check concentrate for crystallization. If crystallization has occurred during storage, warm the wash buffer concentrate to 37°C and mix well to eliminate crystals before removing aliquot for dilution.
  ***Note** - All lots of wash buffer concentrate are interchangeable between assay kits.*
- Working wash buffer containers should be thoroughly cleaned with 70% ethanol and thoroughly rinsed with distilled or deionized water before preparation of the next batch of working wash buffer.

7. REAGENT STORAGE AND HANDLING INSTRUCTIONS

- Store the test components in the refrigerator at 2-8°C away from intense light. Allow them to reach room temperature (20-25°C) before use. Return the test components to the refrigerator after use.
- Do not expose the test components to intense light, direct sunlight, or temperatures above 25°C. Do not freeze the kit.
- When stored as directed, test components will remain stable until expiration dates printed on their labels.

- Keep unused coated strips sealed in their pouches until time for use. Allow the pouch to reach room temperature (20-25°C) before opening it. Return any unused strips to the pouch as soon as possible; seal the pouch with the pouch sealer and refrigerate pouch at 2-8°C.
- After dilution, the working enzyme tracer can be stored for one week at 2-8°C.
- After dilution, the working wash buffer can be stored for one week at 2-8°C.

8. REAGENT INSTABILITY OR DETERIORATION

- The chromogen/substrate may have a slightly blue tinge. If the chromogen/substrate turns a darker blue, it may have become contaminated and should be discarded.
- Any reagent that contains visible particulate matter should be discarded.

9. SPECIMEN COLLECTION AND PREPARATION

- This assay is not designed to test body fluids other than human serum or plasma. This assay is not designed for testing human cadaver fluids.
- Specimens containing precipitate may give inconsistent test results. Do not test specimens containing particulate material, or grossly hemolyzed or lipemic specimens.
- There is a specimen dilutional effect with citrated plasma due to the liquid nature of this anticoagulant. Borderline or high-negative results obtained from citrated specimens should be retested using serum as the matrix.
- Each assay requires 100 µL human serum or plasma. EDTA, citrate or heparin anticoagulants have been tested and may be used with this assay. Follow manufacturer's instructions carefully when using plasma collection containers with anticoagulants.
- The testing of heat inactivated samples is not recommended.
- Samples that are to be used fresh may be stored for up to two hours at 2-8°C in the presence of clots. Serum separated from the clot may be stored at 2-8°C up to 48 hours, but then must be frozen and stored deep-frozen (at –20°C or below) in sterile containers until use (21). If sample is stored frozen, mix thawed sample well before testing. It has been shown that up to three freeze-thaw cycles do not interfere with the assay.
- For shipping, specimens should be frozen at –20°C or below and shipped with dry ice. Temperature level during entire shipment should be no greater (warmer) than –20 °C. Pack specimens in compliance with government regulations covering the transportation of etiologic agents (22).

10. MANUAL ASSAY PROCEDURE

Materials Provided

ETI-MAK-2 PLUS

Coated Strips
Enzyme Tracer
Tracer Diluent
Calibrator (Human)
Negative Control (Human)
Positive Control (Human)
Incubation Buffer
Wash Buffer (Concentrate)
Chromogen/Substrate
Stop Solution
Strip Sealers
Plate Sealers
Pouch Sealer.

Materials Required But Not Provided

Microwell plate washer - The following instrument specifications are recommended for the kit performance:

Volume dispensed: 350-370 µL
Number of wash cycles: 5
Soak time: 30 seconds
Aspirate the last aliquot of dispensed liquid: yes.
***Note** - The volume of each microwell is ca. 400 µL. Make sure the volume of working wash buffer dispensed into each well does not cause the wells to overflow. If the wells overflow, set the washer to dispense less working wash buffer.*

Microwell plate reader - The following instrument specifications are recommended for the kit performance:

Wavelength: dual wavelength, 450 nm and 600-650 nm
Bandwidth: ≤ 10 nm
Absorbance range: 0 absorbance units to ≥ 3 absorbance units
Repeatability: better than or equal to 0.005 absorbance units, or 1%, whichever is greater
Linearity or accuracy: better than or equal to 0.010 absorbance units, or 2%, whichever is greater
Drift: less than 0.005 absorbance units per hour.

Incubator, 37°C ± 1°C.
***Note** - Gravity convection incubators are recommended. Forced-air incubators may cause edge effects. Do not use water baths as incubators.*

Micropipettes with disposable clean tips (50 µL and 100 µL).
***Note** - Suggested specifications for micropipetters (based on gravimetric testing) are:*
50 µL: accuracy ± 3%, precision 2%
100 µL: accuracy ± 2%, precision 1%.

Miscellaneous clean glass or plastic containers
Hazardous waste disposal materials
Disposable gloves
Distilled or deionized water
Pipetter-diluter (optional)
Multichannel pipetter (optional)
Pipette tips for multichannel pipetter (if multichannel pipetter is used)
Disposable reagent reservoirs (if multichannel pipetter is used)
Printer compatible with microwell reader.

Automated Procedure Using Biochem Immunosystems Labotech/ETI-LAB Instrument
See the Labotech (ETI-LAB) instrument Instruction Manual.

Assay Procedure

Perform all assay steps in the order given and without any delays between the steps. A cutoff value is calculated for each plate based on the absorbance values of the calibrators run on that plate. A maximum of one plate should be set up (completed through the first incubation step) at a time. If multiple plates are being run as a batch, each plate must be treated as a single entity; i.e., the calibrators, controls and patient specimens for the plate must be added and the incubation time started before moving on to the next plate. Proper instrument maintenance is critical for good assay performance. Follow the manufacturer's instructions for performing instrument warm-up, quality control, calibration and maintenance procedures on all equipment used in this assay.
Note - All steps must be completed within four hours. Calibrator, positive and negative controls must be run with each plate of patient specimens.

1. **Prepare assay reagents.** Allow all test components to reach room temperature (20-25°C). Prepare working wash buffer and working enzyme tracer according to the directions given in Section 6, Reagent Preparation. Refer to the chart in Section 6 to ensure preparation of sufficient reagent volumes for the number of tests included in the run.

2. **Prepare coated plate.** Prepare enough microwells for the calibrators, controls and patient samples to be tested. Allow one blank well containing only chromogen/substrate and stop solution in well A1. Allow one well for each patient sample. The calibrator must be tested in triplicate and the negative and positive controls tested in singlet. Calibrators are to be placed in wells B1, C1 and D1; negative control is to be placed in well E1; positive control is to be placed in well F1 (for details, refer to the recommended plate map at the end of this section). Test calibrator and controls as you would patient specimens.

Coated strips may be separated. Avoid handling the bottoms of the microwells because scratches or marks could affect the reading of test results. Store unused strips in their original pouch, seal the pouch carefully, and refrigerate at 2-8°C.

3. **Add incubation buffer.** Add **50 µL** incubation buffer to all microwells (except for the blank well).

4. **Add samples and controls.** If sample was stored frozen, mix thawed sample well (vortex) before proceeding. Add **100 µL** of each calibrator, control or sample to its respective microwell. To avoid cross-contamination, use a clean micropipette tip to dispense each calibrator, control or specimen. Record the microwell position of each calibrator, control or patient specimen on a laboratory data sheet.

Incubation buffer is light blue in color. On addition of calibrators, controls or samples, the color will turn green or dark blue. This color change may vary from sample to sample, but it will always be visible.

5. **Incubate.** Cover the microwells with a plate or the strip sealer provided with this kit. Use a roller to affix the sealer or press firmly by hand around microwell and plate edges to ensure that the sealer is firmly attached over the entire strip or plate. Tap the coated plate gently to release any air bubbles trapped in the liquid making sure samples do not splash onto the sealer. Ensure that all microwells are filled equally. Incubate the microwells **for 2 hours ± 10 minutes at 37°C ± 1°C.**

6. **Wash coated plate.** Remove and discard the sealer. Aspirate the liquid from the microwells and wash each well five times as follows: Deliver 350-370 µL of working wash buffer to each microwell, let the wells soak for 30 seconds, and then aspirate the working wash buffer completely from each microwell. Microwell plate washers vary by manufacturer. Make sure the volume of working wash buffer dispensed into each well completely fills the well but does not cause the well to overflow.

7. **Remove excess working wash buffer.** Ensure that all microwells are aspirated completely before proceeding. With some washers it may be necessary to invert the microplate and tap it forcefully on a paper towel to effectively remove residual working wash buffer.

8. **Add working enzyme tracer.** Immediately add **100 µL** working enzyme tracer to each well (except for the blank well).

9. **Incubate.** Cover the microwells with a plate or the strip sealer provided with this kit. Ensure that sealer is applied correctly (see Step 5). Tap the coated plate gently to release any air bubbles trapped in the liquid. Ensure that all microwells are filled equally. Incubate the microwells **for 60 ± 5 minutes at 37°C ± 1°C.**

Warning - Timing of this incubation step is critical.

10. **Wash coated plate.** Remove and discard the sealer. Aspirate the working enzyme tracer from the microwells and wash them as described in Steps 6 and 7.

11. Add chromogen/substrate. Immediately add **100 µL** chromogen/substrate to all microwells, including the blank well.
***Note** - The chromogen/substrate may have a slightly blue tinge. However, if it turns a darker blue, it may have become contaminated and should be discarded.*

12. Incubate. Incubate the microwells **for 30 ± 2 minutes at room temperature** (20-25°C). Avoid exposing the microwells to direct or intense light. Do not exceed the time limits of this incubation.

13. Add stop solution. Add **100 µL** stop solution to each microwell in the same order as chromogen/substrate was added.

14. Read results. Within one hour after addition of stop solution, read the absorbance values of the calibrators, negative control, positive control, and samples with the microwell reader set at 450/630 nm in the bichromatic mode. If time before reading exceeds one hour, the tests must be discarded and specimens retested. Check for and remove air bubbles before reading results. Record the absorbance value for each calibrator, control and sample.
***Note** - Blank the instrument on the blank well. The absorbance of the blank well containing only chromogen/substrate and stop solution (see Step 2 in Section 10, Procedure) is evaluated as described in the QC section. The value for the blank well should be recorded and subtracted from each calibrator, control and sample value before calculating mean values and cutoff, and before interpreting results.*

15. Perform assay quality control procedures. Before evaluating results, perform quality control procedures (see Section 11, Quality Control).

16. Perform equipment quality control and maintenance procedures. Proper instrument maintenance including calibration is critical for good assay performance. Follow the manufacturer's instructions for performing quality control and maintenance procedures on all equipment used in this assay.

Recommended Plate Map

	1	2	3	4	5	6	7	8	9	10	11	12
A	BLK	S3										
B	CAL1	S4										
C	CAL2	S5										
D	CAL3	S6										
E	NC	S7										
F	PC	S8										
G	S1	etc.										S89
H	S2											S90

11. QUALITY CONTROL

The negative and positive controls are intended to monitor for substantial reagent failure. The positive control will not ensure precision at the assay cutoff.

The quality control material furnished is in a serum matrix. It may not adequately control the assay for plasma specimens. The user should provide alternate control material for testing of plasma matrices.

Additional controls may be tested according to guidelines or requirements of local, state, and/or federal regulations or accrediting organizations.

Use the following steps to validate quality control. References 23 and 24 provide guidance on quality control recommendations. Record the results on the QC Verification Worksheet provided for the assay.

Compute the mean absorbance value for the calibrator.

Always evaluate mean calibrator value and negative and positive control values for each plate when running more than one plate in a batch. Be sure to compare the absorbance value of each patient sample with the cutoff value computed for the plate containing that sample.

1. Evaluate the absorbance value of the substrate blank.

Blank the instrument on the blank well containing only chromogen/substrate and stop solution (see Step 14 in Section 10, Procedure). The absorbance value for the blank well must be between 0.000 and 0.150 for the assay to be valid. If the absorbance value of the substrate blank is less than 0.000 or greater than 0.150, the run must be repeated.
Note - Subtract the substrate blank absorbance value from each absorbance value before performing the following evaluations.

2. Evaluate the mean calibrator absorbance value (Cal $\bar{x}$).

Each calibrator absorbance value (after subtraction of the blank) must be greater than –0.020 and less than 0.120.

$$-0.020 < \text{Cal} < 0.120$$

If one of the calibrator absorbance values does not meet this criterion, it should be discarded and the mean value recalculated using the remaining two values. If more than one calibrator absorbance values do not meet this criterion, the run is invalid and must be repeated.

Example 1: Calculation of mean of calibrators

Calibrator well	Absorbance	Minus blank absorbance	Final calibrator absorbance
B1	0.038	0.030	0.008
C1	0.040	0.030	0.010
D1	0.039	0.030	0.009
Total absorbance			0.027

$$\text{Mean of calibrators (Cal } \bar{x}) = \frac{\text{Total absorbance}}{3} = \frac{0.027}{3} = 0.009.$$

The mean calibrator absorbance value must be greater than –0.020 and less than 0.120.

$$-0.020 < \text{Cal } \bar{x} < 0.120$$

If the mean calibrator absorbance value does not meet this criterion, the run is invalid and must be repeated.

3. Evaluate the negative control absorbance value (NC).

After subtracting the substrate blank absorbance, the negative control absorbance value must be greater than –0.020, less than 0.120 and less than the cutoff (CO).

$$-0.020 < NC < 0.120$$
$$NC < CO$$

If the negative control absorbance value does not meet this criterion, the run is invalid and must be repeated.

4. Evaluate the positive control absorbance value (PC).

After subtracting the substrate blank absorbance, the positive control absorbance value must be greater than 0.550 and less than 1.850.

$$0.550 < PC < 1.850$$

If the positive control absorbance value does not meet this criterion, the run is invalid and must be repeated.

5. Evaluate the difference between the positive control absorbance value and the negative control absorbance value.

The difference between the positive control absorbance value and the negative control absorbance value must be greater than 0.500.

$$PC - NC > 0.500$$

If the difference between the positive control absorbance value and the negative control absorbance value does not meet this criterion, the run is invalid and must be repeated.

Example 2: Calculation of difference between PC and NC

Positive control absorbance (PC)	= 1.273
Negative control absorbance (NC)	= 0.010
Difference (PC – NC) = 1.273 – 0.010	= 1.263

12. QUALITY CONTROL PROBLEM SOLVING

It is important to follow the assay procedure precisely. If calibrator or control values are not within acceptable limits (see Section 11, Quality Control) or results differ markedly from those expected, check these assay variables:

- Check incubator, incubation times, and temperatures.
- A properly functioning washer is critical to the assay. Ensure that the washer is filling and aspirating all wells, that no probes are plugged, and that the probes are placed correctly in the microwells. No fluid should be left in the wells at the end of the wash step.
- Be sure that wells do not dry out between washing and addition of the next reagent. Add reagent within a few minutes of removal of the plate from the washer. If a probe (or probes) on the washer becomes plugged during washing, identify the affected microwell(s) but continue with the assay procedure. Retest the affected specimen(s). To unplug probes, refer to the washer operator's manual.
- Check that all reagents and specimens are at room temperature (20-25°C) before starting the assay.

41

- Check that all reagents are within the expiration date, that appropriate assay kit components and ancillaries are used, and that there are no visible signs of contamination such as cloudiness or precipitates.
- Avoid cross-contamination of reagents and wells. If multichannel pipette tips have been contaminated, replace the tips.

13. INTERPRETATION OF RESULTS

The presence or absence of HBsAg is determined by comparing the absorbance values of patient samples with a cutoff value. The cutoff value is determined for each plate based on the absorbance values of the calibrators run on that plate. Be sure to compare the absorbance value of each patient sample with the cutoff value computed for the plate containing that sample.

Calculation of Cutoff Value

The cutoff value is determined for each plate by adding 0.040 to the mean absorbance of the calibrator values after subtraction of the substrate blank.

$$\text{CUTOFF} = \text{Cal } \bar{x} + 0.040$$

Example 3: Calculation of cutoff value

Calibrator mean absorbance	0.009
Constant	+ 0.040
Cutoff value for this run	0.049

The cutoff was established by testing 348 samples (174 volunteer blood donors and 174 hospitalized patients) with three lots of ETI-MAK-2 PLUS. The results were examined as the difference (delta) between single sample absorbance and calibrator absorbance. In the apparently healthy adult (volunteer donor) population, 95% had delta values less than 0.030 and 99% had delta values less than 0.031; in the hospitalized patient population, 95% had delta values less than 0.030 and 99% had delta values less than 0.047.

Interpretation of Results (Manual or Labotech/ETI-LAB assay)

Absorbance Values	Result	Interpretation
Absorbance < 90% x Cutoff	Negative	HBsAg not detected by ETI-MAK-2 PLUS. This result should not be used alone but in conjunction with other hepatitis B serological markers to determine disease state. If specimen has been submitted for prenatal screening confirm result by alternative testing, e.g., other HBV serological marker testing.
Absorbance within 90-110% of Cutoff	Equivocal	Presence of HBsAg indeterminate by ETI-MAK-2 PLUS. Specimen should be retested using ETI-MAK-2 PLUS kit to establish presence or absence of antigen. If a specimen is found repeatedly equivocal, the pattern of other hepatitis B serological markers should be used to identify status of disease, or another sample should be collected and tested at a later date.
Absorbance > 110% x Cutoff	Positive	HBsAg detected by ETI-MAK-2 PLUS. This result should not be used alone but in conjunction with other hepatitis B serological markers to determine disease state. If HBsAg is the only hepatitis B serological marker detected, other

		additional testing should be done to validate the results e.g., testing new patient specimens or testing with an HBsAg confirmatory assay. If specimen is submitted for prenatal screening confirm result by alternative testing, e.g., other HBV serological marker testing.

***Note** - The magnitude of the measured result, above the cutoff, is not indicative of the total amount of antigen present.*

Example 4: Interpretation of results

Cutoff = 0.049
Equivocal zone = 0.044-0.054
Sample No. 1 absorbance = 0.012
Sample No. 2 absorbance = 0.908
Sample No. 1 should be considered negative for HBsAg; sample No. 2 should be considered positive for HBsAg.

14. LIMITATIONS OF THE PROCEDURE

- Results obtained from immunosuppressed patients should be interpreted with caution.
- This assay is not designed to test body fluids other than human serum or plasma.
- The performance characteristics of this assay have not been established for newborn testing.
- Any laboratory test result should be interpreted in conjunction with the patient's clinical presentation and the results of other diagnostic tests. A negative result on a given laboratory assay does not rule out the possibility of infection.
- The prevalence of the analyte will affect the assay's predictive value.
- Assay performance characteristics have not been established when the ETI-MAK-2 PLUS HBsAg assay is used in conjunction with the other manufacturers' assays for specific HBV serological markers. Users are responsible for establishing their own performance characteristics.
- Performance characteristics have not been established for any other automated instrument than the Biochem Immunosystems Labotech/ETI-LAB automated instrument. If another automated instrument is used the user is responsible for establishing their own assay performance characteristics.
- Specimens from patients receiving preparations of mouse monoclonal antibodies for therapy or diagnosis may contain human anti-mouse antibodies (HAMA). Such specimens may produce false negative result when tested with a one-site sandwich immunoassay such as the DiaSorin ETI-MAK-2 PLUS assay. Specimens from these individuals should not be tested with this assay.
- The analytical sensitivity of the DiaSorin MAK-2 PLUS has been determined to be approximately 0.1 PEI U/mL.

15. EXPECTED VALUES

The 236 prospective samples used in the expected values study for the ETI-MAK-2 PLUS assay were from patients who were sent to the laboratory for HBV testing. Of those, 100 (42%) were frozen and 136 (58%) were fresh. The patients represented Florida, Georgia, Pennsylvania,

California, Utah, and the southeastern US. The group was 69% (162/236) female, 29% (68/236) male and 2% (6/236) unspecified; the ethnicity of the patients was unspecified. The ages ranged from 5 to 88 years old, with 6 samples unspecified. The percent DiaSorin ETI-MAK-2 PLUS positive results observed in these samples was 10%.

The table below summarizes the percent ETI-MAK-2 PLUS positive and negative results by gender and age range. There were 6 samples for which gender and age were not reported; they were all positive. There were 6 other samples for which age was not reported, two were from females and four were from males; all were negative. These 12 results were not included in the table.

		DiaSorin ETI-MAK-2 PLUS						
		+		-		E*		TOTAL
Age Range	Gender	n	%	n	%	n	%	
0-9	F	0	0	2	100	0	0	2
	M	0	0	0	0	0	0	0
10-19	F	1	6	16	94	0	0	17
	M	1	50	1	50	0	0	2
20-29	F	3	6	48	94	0	0	51
	M	4	31	9	69	0	0	13
30-39	F	1	2	48	98	0	0	49
	M	3	18	14	82	0	0	17
40-49	F	3	15	16	80	1	5	20
	M	2	14	12	86	0	0	14
50-59	F	1	20	4	80	0	0	5
	M	1	13	7	88	0	0	8
60-69	F	1	33	2	67	0	0	3
	M	0	0	2	100	0	0	2
70-79	F	1	10	9	90	0	0	10
	M	0	0	5	100	0	0	5
80-89	F	1	33	2	67	0	0	3
	M	0	0	3	100	0	0	3
TOTAL		23	10	200	89	1	1	224

High Risk Population

Single repository samples belonging to high-risk populations (66 hemodialyzed patients, 148 hemophiliacs, 150 IV drug users) were tested with the DiaSorin ETI-MAK-2 PLUS assay to determine frequency of positive results in that population. The group was 12% (42/364) female, 69% (252/364) male, and 19% (70/364) unspecified, with ages ranging from 19 to 87 years old. No geographical locations were specified. Equivocal results by the ETI-MAK-2 PLUS assay were repeated if sample volumes permitted. The table below summarizes the ETI-MAK-2 PLUS results. The data in the table represent the number of specimens in each category.

High Risk Population

Population	Frequency of Positive Results (# Positive/Total # Samples)
IV drug users	17/150 = 11.3% (2 equivocal)
Hemophiliacs	12/148 = 8.1%
Hemodialysis patients	0/66 = 0.0%
TOTAL	29/364 = 8.0% (2 equivocal)

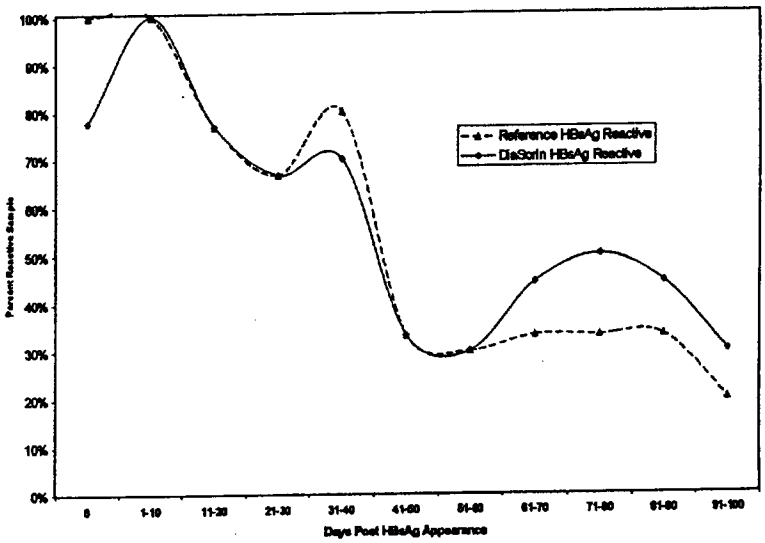
Acute Serial Panels

One hundred twenty-four (124) archived serial samples from nine individuals were tested. Most (8/9) of these individuals were defined as being acutely infected by the appearance of HBsAg and HBeAg with the subsequent appearance of IgM anti-HBc, total anti-HBc, anti-HBe, and anti-HBs. One individual had detectable HBsAg, but did not have detectable HBeAg in any specimen. However, this individual did seroconvert for anti-HBe.

The specimens were collected from individuals undergoing plasmapheresis for further manufacturing purposes. Three individuals were found to be infected with HBV during the first plasmaphereses and others became infected with HBV during subsequent plasmaphereses. It is unknown how long these three initially HBsAg reactives were infected prior to the first plasmaphereses. All nine individuals underwent sequential plasmaphereses after becoming HBV infected. However, the timing of subsequent plasmaphereses varied from individual to individual. The specimens draw times were normalized to represent the day that HBsAg was first detected by an FDA-licensed assay as day 0. Draw days ranged from day 0 (HBsAg first detected) through day 355 post-day 0. Since all panels did not contain the same draw day, sample results were grouped within day intervals (e.g. days 0, 1-10, 11-20, etc., representing days since first detection of HBsAg).

The results are summarized in the following table and graph. In the graph below the pattern for the reference HBsAg percent reactive has been overlaid for reference.

Day Range	Number Speci-mens	DiaSorin HBsAg Reactive	% Positive
0	9	7	77.8%
1-10	10	10	100.0%
11-20	13	10	76.9%
21-30	9	6	66.7%
31-40	10	7	70.0%
41-50	6	2	33.3%
51-60	10	3	30.0%
61-70	9	4	44.4%
71-80	6	3	50.0%
81-90	9	4	44.4%
91-100	10	3	30.0%
101-110	6	1	16.7%
111-120	4	1	25.0%
121-130	4	1	25.0%
131-140	3	0	0.0%
141-150	2	1	50.0%
151-160	0	0	NA
161-170	1	0	0.0%
171-180	0	0	NA
181-190	1	0	0.0%
191-200	1	0	0.0%
355	1	0	0.0%



16. PERFORMANCE DATA

Clinical Samples

Since the majority of studies were performed on preselected retrospective specimens, no calculations for the assay's positive and negative predictive values may be performed or inferred.

Prospective Samples. A study of 136 prospective specimens was conducted. These specimens represented individuals who were sent to the laboratory for hepatitis testing. Specimens were

collected at a reference laboratory and assayed at the California clinical trial site. The patients were 86% (117/136) female and 14% (19/136) male. The ages ranged from 5 to 77 years old, with three specimens not specified.

The study (testing) sites were blinded to the previous specimen categorization. All testing was performed by the manual ETI-MAK-2 PLUS procedure. Specimens were characterized by testing with six HBV serological markers (HBsAg, HBeAg, IgM anti-HBc, total anti-HBc, anti-HBe, anti-HBs) using FDA-licensed or approved assays. Testing with these assays followed the FDA-licensed or approved procedure, including confirmation by neutralization of repeatably reactive HBsAg samples.

Results by Specimen Classification. After study completion all samples were assigned a specimen classification based on the patterns of the six HBV serological markers established by the reference assays. Based on these serological marker patterns, the samples were categorized into the HBV classifications described in the table below. There were six unique HBV marker patterns observed in the ETI-MAK-2 PLUS prospective clinical studies.

Characterization Based On Single Point Specimen	HBsAg	HBeAg	IgM anti-HBc	Total anti-HBc	anti-HBe	anti-HBs	n
Chronic Infection	+	-	-	+	+	-	1
Recovery	-	-	-	+	+	+	2
Past Infection	-	-	-	+	-	+	4
	-	-	-	+	-	-	4
HBV Vaccine Response	-	-	-	-	-	+	38
Not Previously Infected with HBV	-	-	-	-	-	-	87

Based on the above classifications the DiaSorin HBsAg results for the prospective samples were compared to a reference assay's HBsAg results. The following table shows this comparison and percent agreement with 95% confidence intervals with the reference HBsAg assay.

Prospective Samples Comparison

	Reference HBsAg[a]			
	–		+	TOTAL
	ETI-MAK-2 PLUS		ETI-MAK-2 PLUS	
Serological Classification	–	E[b]	+	
Chronic infection	0	0	1	1
Recovery	2	0	0	2
Past infection	8	0	0	8
HBV vaccine response	38	0	0	38
Not previously infected	86	1	0	87
Grand Total	134	1	1	136

[a] Result of initially repeatedly reactive and neutralization testing.
[b] Equivocal results

47

Prospective Samples:		
Chronic Infection	Positive agreement with reference assay results = 100%	(1/1)
	95% CI = 2.5 to 100%	
	Negative agreement with reference assay results = N/A	(0/0)
	95% CI = N/A	
Recovery	Positive agreement with reference assay results = N/A	(0/0)
	95% CI = N/A	
	Negative agreement with reference assay results = 100.0%	(2/2)
	95% CI = 15.8 to 100.0%	
HBV Vaccine Response	Positive agreement with reference assay results = NA	(0/0)
	95% CI = NA	
	Negative agreement with reference assay results = 100%	(38/38)
	95% CI = 90.8 to 100%	
Past Infection	Positive agreement with reference assay results = NA	(0/0)
	95% CI = NA	
	Negative agreement with reference assay results = 100%	(8/8)
	95% CI = 63.1 to 100%	
Not Previously Infected	Positive agreement with reference assay results = NA	(0/0)
	95% CI = NA	
	Negative agreement with reference assay results = 98.9%	(86/87)
	95% CI = 93.8 to 99.9%	

Retrospective Samples. Retrospective studies were carried out at three clinical laboratories in the United States (California, Missouri, and Minnesota) and at DiaSorin (Italy) to assess the performance of the ETI-MAK-2 PLUS assay in detecting HBsAg. The study set included 650 frozen repository samples (the majority of which were purchased from commercial vendors) from the following populations:

- patients with chronic hepatitis B infection (HBsAg positive for greater than six months) - 111 frozen repository samples;
- patients with serologically diagnosed hepatitis B infection (acute, chronic, asymptomatic, convalescent, etc.) - 82 frozen repository samples;
- patients sent to the laboratory for hepatitis B testing - 100 frozen repository samples;
- a general hospital patient population - 357 frozen repository samples.

The specimens represented Midwestern (2%), Southeastern (25%), Western (13%), and Northeastern US (2%), outside of the US (1%), and unspecified (57%). The group was 44% (287/650) female, 42% (270/650) male, and 14% (93/650) unspecified. Approximately 13% (84/650) were Caucasian, 4% (27/650) were African American, < 1% (5/650) were Hispanic, < 1% (3/650) were Asian, and 82% (531/650) were unspecified. The ages ranged from 5 to 98 years old, with 131 specimens not specified.

The study (testing) sites were blinded to the previous specimen categorization. All testing was performed by the manual ETI-MAK-2 PLUS procedure. Specimens were characterized by testing with six HBV serological markers (HBsAg, HBeAg, IgM anti-HBc, total anti-HBc, anti-HBe, anti-HBs) using FDA-licensed or approved assays. Testing with these assays followed the FDA-licensed or approved procedure with the exception of the HBsAg assay at two of the three sites. At these sites, the majority of specimens that were initially HBsAg-positive were repeated in duplicate, however the repeatedly reactive specimens were not confirmed by the licensed HBsAg confirmation assay at the two sites. Therefore, true HBsAg result was determined in one of three ways: 1) confirmed by reference assay neutralization during clinical trials, 2) based on a statement by the attending physician that HBsAg was positive for greater than 6 months, or 3) information provided by the

vendor regarding confirmatory testing performed at their location or by the material source facility. Ten samples were excluded from analysis (4 hospital patients and 6 patients that came to the lab for HBV testing) because the true HBsAg result for these samples was not determined by one of these three methods.

Results by Specimen Classification. After study completion all samples were assigned a specimen classification based on the patterns of the six HBV serological markers established by the reference assays. Based on these serological marker patterns, the samples were categorized into the HBV classifications described in the table below. There were 35 unique HBV marker patterns observed in the ETI-MAK-2 PLUS retrospective clinical studies.

Characterization Based On Single Point Specimen	HBsAg	HBeAg	IgM anti-HBc	Total anti-HBc	anti-HBe	anti-HBs	n
Acute infection	+	+	+ or I*	+	-	-	52
	+	-	+ or I	+	+	-	4
	+	-	-	-	-	-	2
	+	+	-	-	-	-	2
Chronic Infection	+	-	-	+	+	-	82
	+	+	-	+	-	-	21
	+	-	-	+	- or I	-	23
	+	+	+	+	-	+	4
	+	+	- or I	+	-	+	2
	+	-	-	+	+	+	2
	+	+	-	+	+ or I	+	2
	+	+	+	+	+	+	1
	+	+	-	+	+	-	1
	+	-	-	+	-	+	1
Recovery	-	-	-	+	+ or I	+	40
	-	-	-	+	+	-	6
	-	-	+	+	+	-	2
	-	-	+ or I	+	+	+	2
Past Infection	-	- or I	-	+	-	+	12
	-	-	-	+	-	-	9
HBV Vaccine Response	-	-	-	-	-	+	20
Not Previously Infected with HBV	-	-	-	-	-	-	343
Uninterpretable	-	+ or I	-	-	-	-	13
	-	+	-	+	-	+	2
	-	+	-	+	+	+	1
	-	I	-	+	-	-	1

*I = Indeterminate result.

Based on the above classifications the ETI-MAK-2 PLUS HBsAg results for the retrospective samples were compared to a reference assay's HBsAg results as determined by the methods described above. The following tables show this comparison and percent agreement with 95%

confidence intervals with the reference HBsAg results. The data are presented in three tables based on the means of determining true HBsAg results.

Retrospective Samples Comparison – True HBsAg Result Based on Statement from Physician

Reference Serology Classification	Reference HBsAg		TOTAL
	–	+	
	ETI-MAK-2 PLUS	ETI-MAK-2 PLUS	
	–	+	
Acute infection	0	26	26
Chronic infection	0	61	61
Recovery	1	0	1
Not previously infected	1	0	1
Grand Total	2	87	89

Acute Infection

Positive agreement with reference assay results = 100% (26/26)
95% CI = 86.8 to 100%
Negative agreement with reference assay results = NA (0/0)
95% CI = NA

Chronic Infection

Positive agreement with reference assay results = 100.0% (61/61)
95% CI = 94.1 to 100.0%
Negative agreement with reference assay results = NA (0/0)
95% CI = NA

Recovery

Positive agreement with reference assay results = NA (0/0)
95% CI = NA
Negative agreement with reference assay results = 100% (1/1)
95% CI = 2.5 to 100%

Not Previously Infected

Positive agreement with reference assay results = NA (0/0)
95% CI = NA
Negative agreement with reference assay results = 100.0% (1/1)
95% CI = 2.5 to 100.0%

Retrospective Samples Comparison - True HBsAg Result Based on Confirmation by Neutralization During Clinical Trials

Reference Serology Classification	Reference HBsAg				TOTAL
	–		+		
	ETI-MAK-2 PLUS		ETI-MAK-2 PLUS		
	–	+	–	+	
Acute infection	0	0	0	25	25
Chronic infection	0	0	1	47	48
Recovery	16	0	0	0	16
Past infection	6	0	0	0	6
HBV vaccine response	6	0	0	0	6
Not previously infected	110	3	0	0	113
Uninterpretable	5	2	0	0	7
Grand Total	143	5	1	72	221

Acute Infection

Positive agreement with reference assay results = 100% (25/25)
95% CI = 86.3 to 100%
Negative agreement with reference assay results = NA (0/0)
95% CI = NA

Chronic Infection

Positive agreement with reference assay results = 97.9% (47/48)
95% CI = 88.9 to 99.9%
Negative agreement with reference assay results = NA (0/0)

		95% CI = NA
Recovery	Positive agreement with reference assay results = NA	(0/0)
		95% CI = NA
	Negative agreement with reference assay results = 100%	(16/16)
		95% CI = 79.4 to 100%
HBV Vaccine Response	Positive agreement with reference assay results = NA	(0/0)
		95% CI = NA
	Negative agreement with reference assay results = 100.0%	(6/6)
		95% CI = 54.1 to 100.0%
Past Infection	Positive agreement with reference assay results = NA	(0/0)
		95% CI = NA
	Negative agreement with reference assay results = 100%	(6/6)
		95% CI = 54.1 to 100%
Not Previously Infected	Positive agreement with reference assay results = NA	(0/0)
		95% CI = NA
	Negative agreement with reference assay results = 97.3%	(110/113)
		95% CI = 92.4 to 99.4%
Uninterpretable	Positive agreement with reference assay results = NA	(0/0)
		95% CI = NA
	Negative agreement with reference assay results = 71.4%	(5/7)
		95% CI = 29.0 to 96.3%

Retrospective Samples Comparison – True HBsAg Result Based on Vendor Information

Reference Serology Classification	Reference HBsAg				TOTAL
	–		+		
	ETI-MAK-2 PLUS		ETI-MAK-2 PLUS		
	–	+	+	E*	
Acute infection	0	0	6	0	6
Chronic infection	0	0	22	1	23
Recovery	33	0	0	0	33
Past infection	15	0	0	0	15
HBV vaccine response	13	1	0	0	14
Not previously infected	222	7	0	0	229
Uninterpretable	9	1	0	0	10
Grand Total	292	9	28	1	330

* Equivocal results

Acute Infection	Positive agreement with reference assay results =	100% (6/6)
		95% CI = 54.1 to 100%
	Negative agreement with reference assay results =	NA (0/0)
		95% CI = NA
Chronic Infection	Positive agreement with reference assay results =	100.0% (22/22)
		95% CI = 84.6 to 100.0%
	Negative agreement with reference assay results =	NA (0/0)
		95% CI = NA
Recovery	Positive agreement with reference assay results =	NA (0/0)
		95% CI = NA
	Negative agreement with reference assay results =	100% (33/33)
		95% CI = 89.4 to 100%
HBV Vaccine Response	Positive agreement with reference assay results =	NA (0/0)
		95% CI = NA
	Negative agreement with reference assay results =	92.9% (13/14)
		95% CI = 66.1 to 99.8%

Past Infection	Positive agreement with reference assay results =	NA	(0/0)
	95% CI =	NA	
	Negative agreement with reference assay results =	100%	(15/15)
	95% CI =	78.2 to 100%	
Not Previously Infected	Positive agreement with reference assay results =	NA	(0/0)
	95% CI =	NA	
	Negative agreement with reference assay results =	96.9%	(222/229)
	95% CI =	93.8 to 98.8%	
Uninterpretable	Positive agreement with reference assay results =	NA	(0/0)
	95% CI =	NA	
	Negative agreement with reference assay results =	90.0%	(9/10)
	95% CI =	55.5 to 99.7%	

Samples from Pregnant Women: Single samples collected from pregnant women (154 prospective samples and 410 retrospective samples) were tested with both DiaSorin and reference HBsAg assays. Positive results from the retrospective samples were confirmed by reference method neutralization at DiaSorin Italy after completion of the trials. Nonreactive results from the prospective samples were verified by testing for HBeAg, anti-HBe, total anti-HBc and IgM anti-HBc at the trial site. All nonreactive specimens were HBeAg and IgM anti-HBc nonreactive confirming the nonreactive HBsAg. The one positive sample from the prospective population was repeat tested for HBsAg and confirmed by reference method neutralization at the trial site. The table below compares the ETI-MAK-2 PLUS results with the HBsAg reference assay. The data in the table represent the number of specimens in each group. Equivocal results by the ETI-MAK-2 PLUS were repeated per the insert instructions, if sample volumes permitted and the repeat results used in the calculation.

Pregnant Women – Samples Comparison

Group	Reference HBsAg					Total
	–		+			
	ETI-MAK-2 PLUS		ETI-MAK-2 PLUS			
	–	+	–	+	E*	
Prospective Samples	154	0	0	0	0	154
Retrospective Samples	378	12	1	17	2	410
Total	532	12	1	17	2	564

* Equivocal results

Pregnant Women Samples

Prospective Samples:

Percent Positive Agreement =	0.0% (0/0)	95% CI = N/A
Percent Negative Agreement =	100.0% (154/154)	95% CI = 97.6 to 100.0

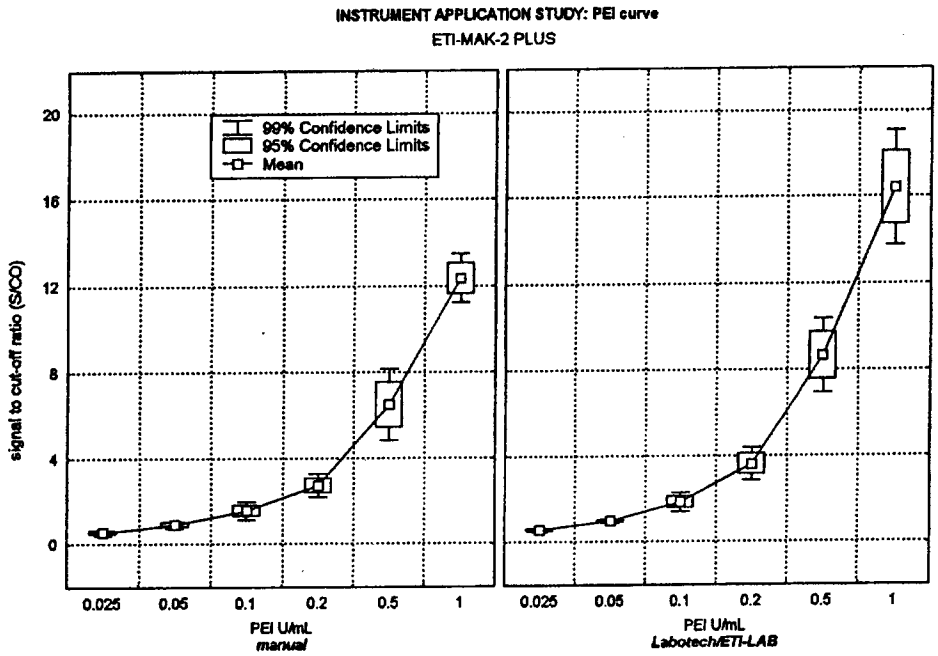
Retrospective Samples:

Percent Positive Agreement =	85.0 (17/20)	95% CI = 62.1 to 96.8%
Percent Negative Agreement =	96.9% (378/390)	95% CI = 94.7 to 98.4

Instrument Comparison of Biochem Immunosystems Labotech/ETI-LAB to the Manual Method: An instrument application study was conducted at DiaSorin, Saluggia Italy, to evaluate the performance of the ETI-MAK-2 PLUS assay on the Biochem Immunosystems Labotech/ETI-LAB, an automated microplate processing instrument, compared to the manual analysis. The Paul-Ehrlich-Institut (PEI) Standard, 12 serum samples near the ETI-MAK-2 PLUS cutoff and samples from the

clinical trials (32 suspected hepatitis B patients and 8 apparently healthy adults) were tested in parallel manually and on the Labotech.

Serial dilutions of the PEI Standard were prepared in fetal calf serum to obtain a panel ranging from high concentration to below the analytical sensitivity of the assay. The diluted Standard samples were tested in duplicate, one run per day for three days both manually and on the Labotech. Due to the requirement that assay cutoff be established for each plate, reproducibility was evaluated based on specimen absorbance-to-cutoff ratios (S/CO) rather than absolute absorbance values. The 95% confidence intervals were established for the S/CO values of each point of the Standard-referenced curve and therefore the analytical endpoint sensitivity was defined (first dilution with S/CO > 1.1). A graph summarizing these results is presented below:



The 12 samples near the cutoff were tested in triplicate, one run per day for three days both manually and on the Labotech. The samples from the clinical trials were tested in singlet in one run on one day, both manually and on Labotech. The mean, the standard deviation and the coefficient of variation (CV%) of the S/CO values were computed by the different components of variability for each of the tested specimens. A summary of the data is presented in the following table.

	Manual			Labotech/ETI-LAB		
Analytical Endpoint Sensitivity (0.1 PEI U/mL)	Mean	W/R %CV[a]	D/D %CV	Mean	W/R %CV	D/D %CV
S/CO [95% CI][b]	1.56 [1.29 – 1.82]	11.8	15.1	1.84 [1.56 – 2.12]	4.1	15.7
12 Cutoff Samples S/CO	1.21	11.2	7.9	1.43	4.0	13.0
Range of mean S/CO	0.91 – 1.44			1.11 – 1.80		
Clinical Samples:						

Suspected Hepatitis B Range of S/CO	Negative: N/A (0/32) Equivocal: N/A (0/32) Positive: 23.9 – >39 (32/32)	Negative: N/A (0/32) Equivocal: N/A (0/32) Positive: 35.5 – >62 (32/32)
Healthy Adults Range of S/CO	Negative: 0.24 – 0.64 (8/8) Equivocal: N/A (0/8) Positive: N/A (0/8)	Negative: 0.08 – 0.46 (8/8) Equivocal: N/A (0/8) Positive: N/A (0/8)

[a] %CVs were calculated using specimen absorbance-to-cutoff ratios (S/CO) which normalized the data plate-to-plate
[b] 95% CI = 95% Confidence Interval; W/R = within-run; D/D = day-to-day

Reproducibility

Manual Assay. Intra-assay, inter-assay, inter-lot, and inter-site variability studies were carried out on the ETI-MAK-2 PLUS kit to test the variability within runs, between runs, between days, between kit lots, and between test sites. Variability was measured on a panel of ten sera that included negative, borderline, and positive samples. Three ETI-MAK-2 PLUS kit lots were tested at three independent test sites. Due to the requirement that assay cutoff be established for each plate, reproducibility was evaluated based on specimen absorbance to cutoff ratios (S/CO) rather than absolute absorbance values. The results of that study are tabulated below.

Clinical Site Reproducibility Study

ID#		# of Tests per Sample	Mean S/CO's	Within-run %CV*	Between-run %CV	Between-lot %CV	Between-day %CV	Between-site %CV	Total
S01	High	108	13.09	6.08	12.11	10.63	9.70	9.03	16.51
S02	High	108	5.59	4.44	10.89	7.82	11.60	8.98	17.50
S03	Low	108	3.19	4.51	11.45	12.04	7.76	9.33	18.66
S04	Low	108	2.70	7.47	8.14	12.65	8.61	6.72	20.18
S05	Low	108	3.67	7.81	8.17	7.24	9.71	8.04	14.83
S06	Low	108	2.57	4.40	17.13	12.58	16.81	7.31	25.88
S07	Equiv	108	1.33	5.37	13.09	10.89	14.11	15.62	29.05
S08	Equiv	108	0.96	9.19	10.09	13.48	8.82	5.00	24.04
S09	Equiv	108	1.01	15.66	13.89	16.93	20.65	3.66	32.00
S10	Neg	108	0.22	15.71	29.33	30.67	59.12	21.18	104.13

* %CVs were calculated using specimen absorbance-to-cutoff ratios (S/CO) which normalized the data plate-to-plate

Plasma Reproducibility. A plasma reproducibility study was conducted at DiaSorin, Saluggia Italy, to evaluate the performance of the manual ETI-MAK-2 PLUS assay on serum versus a variety of plasma types. The plasma types evaluated were citrate, heparin and EDTA. Sample sets of matched serum/multiple plasma were used in the study. A sample set was prepared by spiking the same high-positive sample into each of the matrices (serum and plasmas) resulting in a total of four specimens per set around the cutoff. Several high-positive samples were used in the preparation of the 12 different near-cutoff sample sets. Six matched serum/multiple plasma samples sets were tested in triplicate in each run; thus there were two runs per day for three days, all tested in a manual mode. Due to the requirement that assay cutoff be established for each plate, reproducibility was evaluated based on specimen absorbance to cutoff ratios (S/CO) rather than absolute absorbance values. The mean, the standard deviation and the coefficient of variation (CV%) of the S/CO values were computed by the different components of variability for each of the tested specimens. The 95% confidence intervals were established for the S/CO values of all serum samples and each plasma type. A summary of the data is presented in the following table.

	Serum	Citrate	Heparin	EDTA
Mean S/CO	0.91	0.92	0.87	0.91
95% CI*	0.87-0.94	0.88-0.96	0.83-0.91	0.88-0.95
W/R %CV**	6.3%	6.9%	7.1%	7.8%
D/D %CV	8.1%	9.6%	6.7%	8.0%
Total %CV	9.9%	11.6%	9.6%	10.0%
Between matrix %CV: 8.5%				
Across matrix total %CV: 13.1%				

* 95% CI = 95% Confidence Interval; W/R = within-run; D/D = day-to-day
** %CVs were calculated using specimen absorbance-to-cutoff ratios (S/CO) which normalized the data plate-to-plate

Assay reproducibility using the Labotech has not been established. If the Labotech is used, the user should establish appropriate assay reproducibility in accordance with NCCLS EP5-A, Evaluation of Precision Performance of Clinical Chemistry Devices.

Analytical Sensitivity

The analytical sensitivity of the assay (the smallest quantity of analyte that can be distinguished from background) was evaluated using single point serial dilutions of a standard preparation from the Paul-Ehrlich-Institut (PEI). The analytical sensitivity of the assay (last positive dilution) was determined to be 0.1 PEI U/mL (Mean Signal-to-Cutoff Ratio = 1.56; 95% Confidence Interval = 1.29 to 1.82).

Cross-Reactivity

Of the 525 potential interfering samples, 477 (91%) were negative and 48 (9%) were positive by ETI-MAK-2 PLUS. Among the 48 positive samples, 12 were negative for HBeAg and IgM anti-HBc and negative for HBsAg on repeat testing; 36 were positive by reference testing or review of hepatitis B marker patterns for those samples. As expected, individuals infected by HDV are also infected with HBV. Disease was determined by serological testing, there is no guarantee that the associated antigen was present in the tested material. Interference testing with the described specimens was not performed.

Cross-Reactivity Study Results

GROUP	n	ETI-MAK-2 PLUS Negative or Equivocal Samples	ETI-MAK-2 PLUS Positive Samples	% Confirmed Positive By Additional Testing
Acute EBV infection	16	13	3[a]	–
Acute CMV infection	20	17	3[a]	–
Acute HSV infection	10	10	0	–
Acute Toxoplasma infection	18	18	0	–
Acute parvovirus B19 infection	5	5	0	–
HTLV-I/II infection	50	47	3[b]	100% (1/1)
Syphilis	26	25	1[a]	–
HCV infection	50	48	2[a]	–

GROUP	n	ETI-MAK-2 PLUS Negative or Equivocal Samples	ETI-MAK-2 PLUS Positive Samples	% Confirmed Positive By Additional Testing
HDV infection	20	1	19	100% (19/19)
HIV infection	50	50	0	–
Acute HAV infection	50	47	3	100% (3/3)
Past HAV infection	50	44	6[c]	100% (5/5)
Rheumatoid factor (RF) +	40	40	0	–
Autoimmune disease, including SLE	30	30	0	–
Autoimmune hepatitis	5	5	0	–
Myeloma	20	20[d]	0	–
Hypergammaglobulinemia	20	20	0	–
Influenza vaccine	5	5	0	–
Elevated liver enzymes	10	9	1	100% (1/1)
Non-viral liver disease	30	23	7	100% (7/7)
TOTAL	525	477 (91%)	48 (9%)	100% (36/36)

[a] These samples were negative for HBeAg and IgM anti-HBc, and negative on repeat testing on ETI-MAK-2 PLUS.
[b] 2 samples were negative for HBeAg and IgM anti-HBc, and negative on repeat testing on ETI-MAK-2 PLUS.
[c] 1 sample was negative for HBeAg and IgM anti-HBc, and negative on repeat testing on ETI-MAK-2 PLUS; 5 samples were positive by reference testing.
[d] 1 sample was repeatedly equivocal and all other markers were negative.

A BLAST analysis [Basic Local Alignment Search Tool, National Center for Biotechnology Information, National Institutes of Health, http://www.ncbi.nlm.nih.gov/BLAST] was performed to determine if untested viral or bacterial proteins could potentially cross-react with the anti-HBs monoclonal antibodies used in this assay. No notable similarities in protein sequences were identified from these viral or bacterial proteins, suggesting that they should not cross-react in this assay.

Substances That Do Not Interfere

As recommended by NCCLS Protocol EP7 (25), the ETI-MAK-2 PLUS assay was evaluated for interference by testing the following substances. Testing was performed using matched pairs of negative donor serum and negative donor serum spiked with high-titer HBsAg samples to obtain a result near the cutoff. None of the compounds at the levels indicated were found to interfere with the clinical interpretation of the assay in serum. No interference was found with bilirubin in plasma (EDTA, heparin or citrate), testing for interference with hemoglobin and triolein was not performed in plasma.

Compound	Concentration	
Bilirubin	0.18 mmol/L	10 mg/dL
Hemoglobin	0.06 mmol/L	100 mg/dL
Triolein	33.9 mmol/L	3000 mg/dL

The ETI-MAK-2 PLUS assay was also evaluated for possible interference from heterophilic anti-mouse antibodies (HAMA). A dilutional panel was used, consisting of 21 samples prepared from a

stock pool of high positive human serum. The HAMA concentrations in the samples ranged from 0 to 2975.5 ng/mL, as determined by a HAMA ELISA. In a direct non-competitive assay, such as ETI-MAK-2 PLUS, interference would manifest as false negative results. No interference was seen in that all 21 dilutions were negative by the ETI-MAK-2 PLUS assay, including the HAMA negative panel member.

17. ABBREVIATED TEST PROCEDURE

1. DISPENSE 50 µL INCUBATION BUFFER.
2. DISPENSE 100 µL CALIBRATOR, CONTROLS AND SAMPLES INTO WELLS, LEAVING AN EMPTY WELL FOR THE BLANK.
3. INCUBATE FOR TWO HOURS AT 37°C.
4. ASPIRATE THE LIQUID. WASH THE WELLS REPEATEDLY WITH WORKING WASH BUFFER.
5. DISPENSE 100 µL ENZYME TRACER TO EACH WELL.
6. INCUBATE FOR 60 MINUTES AT 37°C.
7. ASPIRATE THE LIQUID. WASH THE WELLS REPEATEDLY WITH WORKING WASH BUFFER.
8. DISPENSE 100 µL CHROMOGEN/SUBSTRATE TO EACH WELL.
9. INCUBATE FOR 30 MINUTES AT ROOM TEMPERATURE.
10. DISPENSE 100 µL STOP SOLUTION TO EACH WELL.
11. READ THE ABSORBANCE VALUES WITH A PHOTOMETER AT 450/630 nm WITHIN 60 MINUTES.

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