

Laboratory Procedure Manual

Analyte: **Hepatitis B Surface Antigen (HBsAg)**

Matrix: **Serum**

Method: **Auszyme Monoclonal**

Method No.:

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as performed by: Hepatitis Branch
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Important Information for Users

The National Center for Infectious Diseases periodically refines these laboratory methods. It is the responsibility of the user to contact the person listed on the title page of each write-up before using the analytical method to find out whether any changes have been made and what revisions, if any, have been incorporated.

Public Release Data Set Information

This document details the Lab Protocol for testing the item listed in the following table:

File Name	Variable Name	SAS Label
HEPBD_d	LBXHBG	Hepatitis B surface antigen

1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

Sensitive enzyme immunoassays used to detect the presence of HBsAg were first described by Engvall and Perlmann (1–3) and VanWeemen and Schuur (4) in 1971. In 1976 and 1977, solid-phase “sandwich” enzyme immunoassays for the detection of HBsAg were described by Wisdom (5), Wolters *et al.*, (6) and Wei *et al.* (7). The production, characterization and application of monoclonal antibodies for the detection of HBsAg has previously been reported (8–13).

Specimens nonreactive by the Auszyme Monoclonal tests are considered negative for HBsAg and need not be tested further. All specimens considered reactive initially should be repeat tested in duplicate using the same procedure as that used in the initial test. If neither of the repeat tests is reactive, the specimen should be considered negative for HBsAg. If the specimen is reactive in either of the repeat tests, the sample should be considered repeatedly reactive.

2. SAFETY PRECAUTIONS

EIA test kits for HBsAg contain components derived from human serum or plasma. Although various treatments in the manufacturing process are sufficient to inactivate most blood-borne pathogens, there is no assurance that these reagents are entirely noninfectious. Therefore, treat components of test kits as though they are capable of transmitting disease. Furthermore, all serum specimens for analysis should be considered potentially positive for infectious agents including HIV and the hepatitis B virus. Observe universal precautions; wear protective gloves, eye wear, and lab coat during all steps of this method because of infectious contamination hazards. Place all plastic and glassware contaminated with serum in a plastic autoclave bag for disposal. Keep these bags in appropriate containers until sealed and autoclaved. Wipe down all work surfaces with 10% bleach solution when work is finished. Biosafety Level 2 containment and practice as described in CDC/NIH publication #88-8395 are recommended for handling test specimens and kit reagents (14).

Material safety data sheets (MSDSs) are available through the National Center for Infectious Diseases (NCID) computer network. Risk is minimal because of the small quantity of chemicals, the packaging of the chemicals, and limited handling required by the operators using the test kits.

3. COMPUTERIZATION; DATA SYSTEM MANAGEMENT

- A. Raw data are transcribed manually from an instrument readout sheet into a computerized database. This database was custom-designed for the management of CDC Hepatitis Reference Laboratory (HRL) test results, and functions within SQL Server software (Microsoft, Redmond, WA) with a Visual Basic (Microsoft, Redmond, WA) user interface. Test values are compared with a cutoff value calculated from the controls. Results are expressed as "positive" or "negative" Other information in the database may typically include the HRL identification number, the specimen number, the date collected, the date tested and results of testing for other hepatitis markers. Reporting is done directly from the database in printed form or by electronic transfer. Electronically stored data are backed up routinely.
- B. Finished data are reviewed by the lab supervisor. After each NHANES container is completed (i.e., when all clinical evaluations and analyses from each mobile survey site are complete), the supervisor will transmit the results to the SQL Server along with other NHANES IV data.
- C. Files stored on the CDC Local Area Network (LAN) are automatically backed up nightly to tape by CDC Data Center staff.
- D. Documentation for data system maintenance is maintained with printed copies of data records for 2 years.

4. SPECIMEN COLLECTION, STORAGE, AND HANDLING PROCEDURES; CRITERIA FOR SPECIMEN REJECTION
 - A. Specimens submitted for testing are handled according to the HRL SOP entitled "Sample Handling" (W. Kuhnert, 10/02).
 - B. No special instructions such as fasting or special diets are required. Diurnal variation is not a major consideration.
 - C. Specimens may be serum, recalcified plasma, or plasma. Serum specimens may be collected using regular red-top or serum-separator Vacutainers.
 - D. Required sample volume is 10 μ L for the assay; 1.0 mL will permit repeat analyses as well as other testing.
 - E. Specimens should be stored in plastic vials and sealed tightly to prevent desiccation of the sample.
 - F. Serum or plasma samples are collected aseptically to minimize hemolysis and bacterial contamination.
 - G. Samples are stored in labeled 2 mL Nalgene cryovials or equivalent.
 - H. Serum is best stored frozen, and freeze/thaw cycles should be kept to a minimum. Store samples at 4–8°C for no more than 5 days.
 - I. For storage >5 days, samples are held at –20°C. Samples held in long-term storage at –20°C are indexed in the database for easy retrieval.
 - J. Specimens are rejected if contaminated, hemolyzed, or stored improperly. However, rejection is done only after consultation with NCHS.
 - K. Avoid multiple freeze/thaw cycles.
 - L. Do not use heat-activated specimens.
 - M. Performance has not been established for cadaver specimens or body fluids other than serum or plasma (such as urine, saliva or pleural fluid.)
5. PROCEDURES FOR MICROSCOPIC EXAMINATIONS; CRITERIA FOR REJECTION OF INADEQUATELY PREPARED SLIDES

Not applicable for this procedure.
6. EQUIPMENT AND INSTRUMENTATION, MATERIALS, REAGENT PREPARATION, CALIBRATORS (STANDARDS), AND CONTROLS
 - A. Instrumentation
 - (1) Abbott QWIKWASH, model 6258-27 (Abbott Laboratories, North Chicago, IL).
 - (2) Abbott COMMANDER dynamic incubator, model 6210-01, set at 40°C for incubations (Abbott Laboratories).
 - (3) Abbott QUANTAMATIC spectrophotometer, model 7553, set for reading at 492 nm (Abbott Laboratories).
 - (4) Gilson Pipetman micropipettors, 10- and 200- μ L sizes (Rainin Instrument Co., Woburn MA).

B. Materials

- (1) Auszyme Monoclonal, cat. no. 0198024 (Abbott Laboratories).
- (2) Reaction trays, Costar cat. no. 4870 (VWR Scientific, Bridgeport, CT).
- (3) Cover seals provided as part of the anti-HAV test kit (Abbott Laboratories).
- (4) Deionized water (Continental Water Systems, Inc., San Antonio, TX).
- (5) N sulfuric acid, cat. no. 7212 (Baxter).
- (6) Pipette tips, cat. nos. RT20 & RT200 (Rainin Instrument Co.).
- (7) Protective gloves, small/medium/large (any vendor).
- (8) 2-mL cryovials, cat. no. 5000-0020 (Nalge Company, Inc., Rochester, NY).
- (9) Cryovial boxes, cat. no. 5026-0909 (Nalge).
- (10) 1.5-mL microtubes (Marsh Biomedical Products, Rochester, NY).
- (11) 50-mL polypropylene tubes (Corning Glass Works, Corning, NY)
- (12) 5.25% sodium hypochlorite, household bleach (any vendor).

C. Reagent Preparation

- (1) Reagents for these procedures are prepared by the manufacturer of the test kits. Volumes are for 100-test kit sizes.
 - (a) 100 Anti-HBs (mouse) monoclonal coated beads. Antibody to hepatitis B surface antigen (mouse monoclonal).
 - (b) 1 vial (5 ml) Auszyme (mouse) monoclonal conjugate. Antibody to hepatitis B surface antigen (mouse monoclonal): Peroxidase (horseradish). Minimum concentration: 0.2 µg/ml in TRIS buffer with protein stabilizers. Preservative: gentamicin sulfate and thimerosal. Dye: Red No. 33.
 - (c) 1 vial (6 ml) positive control. Human HBsAg, 9 ± 2 ng/ml in TRIS buffer with protein stabilizers. Preservative: gentamicin sulfate and thimerosal. Dye: bromophenol blue.
 - (d) 1 vial (9 ml) negative control. Recalcified human plasma, non-reactive for HBsAg, and anti-HBs. Preservative: gentamicin sulfate and thimerosal.
 - (e) Diluent for α -phenylenediamine•2HCL (OPD); 1 bottle (55 mL). Citrate-phosphate buffer containing 0.02% (v/v) hydrogen peroxide
 - (f) α -phenylenediamine•2HCL (OPD). 1 bottle (10 tablets), each 12.8 mg.
- (2) OPD substrate solution - Bring OPD reagents to 20–25°C. Five to 10 min prior to color development, prepare the OPD substrate solution by dissolving the OPD tablets in diluent for OPD. Using clean pipettes and metal-free containers, transfer the solution into a suitable container 5 mL of diluent for OPD for each tablet to be dissolved. Transfer an appropriate number of OPD tablets into a measured amount of diluent using nonmetallic forceps or equivalent. Allow the tablets to dissolve. *The OPD substrate solution must be used within 60 min of preparation*

and must not be exposed to strong light. Just prior to dispensing the solution for color development, swirl the container gently to obtain a homogeneous solution. Remove air bubbles from the dispenser tubing and prime dispenser prior to use.

D. Standards Preparation

No calibration curve is generated by the user as part of this assay method. Positive and negative control reagents are supplied with each test kit. The assay cutoff value is calculated from values obtained from these controls.

E. Preparation of Quality Control Materials

This method does not employ conventional calibrators or standards. Calibration is based on the results of defined "positive" and "negative" controls.

(1) Kit positive and negative controls

Supplied ready-to-use by the manufacturer of the test kits, Abbott Laboratories.

(a) Negative control

1 vial (9 mL), recalcified human plasma, non-reactive for HBsAg and anti-HBs.

(b) Positive control (human)

1 vial (6 mL), Human HBsAg, 9 ± 2 ng/ml in TRIS buffer with protein stabilizers.
Preservative: gentamicin sulfate and thimerosal. Dye: bromophenol blue.

(2) In-house control (IHC)

Purchased from Blackhawk Biosystems (Virotrol I) and included each time the assay is run. Each lot is validated with multiple runs to establish a valid range. Each time the control is run it must fall within the calculated range to be valid; if the control does not fall within this range the run will not pass validation.

7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

A. Calibration Curve

No calibration curve is generated by the user as part of these assay methods. Calibration of instruments is either automatic or performed periodically by contracted service personnel.

B. Verification

The instrument used to read assay results is equipped to analyze the positive and negative controls for each test series. If, within a testing series, positive or negative controls do not conform to specifications as defined in the product literature, the entire series is invalidated by the instrument.

8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

A. Preliminaries

(1) Reagents are used per kit of 100. Kit components are occasionally interchanged within a manufacturer's lot, but never interchanged between lots.

(2) Remove the test kit from 4–8°C storage. Allow 30–40 min for the reagents to warm to 20–25°C. Swirl gently before use.

(3) Assay 3 negative controls, 2 positive controls, and the in-house control with each run.

- (4) Ensure that all reaction trays are subjected to the process and incubation times that are specified in the manufacturer's instructional literature.
- (5) Once the assay has been started, complete all subsequent steps without interruption and within the recommended time limits.

B. Sample Preparation

- (1) Bring the serum specimens to 20–25°C. Serum and plasma samples may settle into layers when frozen or stored at 4–8°C for extended periods. Mix them gently before testing.
- (2) Label the reaction tray wells for each specimen or control.

C. Instrument Setup

- (1) Operation of the Abbott QWIKWASH

The Abbott QWIKWASH is a semi-automated instrument that is used to wash the beads of the Abbott immunoassays between reagent steps. The wash solution is deionized water.

- (a) Turn on the QWIKWASH using the toggle switch on the back of the instrument. The vacuum pump will come on, as will the "Power" indicator on the instrument.
- (b) Ensure that the "Low Pressure" and "Low Water Level" indicators are NOT illuminated before washing beads. See note below.
- (c) Place the bead tray on the QWIKWASH with the first row of beads aligned with the washing heads.
- (d) Push down on the handle on the top of the instrument. The beads will automatically go through one wash cycle, which will take about 4 sec.
- (e) Raise the handle and slide the tray over until the second row of beads is aligned with the washing heads.
- (f) Repeat until all of the beads have been washed. Then proceed directly to the next step of the assay procedure.

NOTE: The wash water is held in a stainless-steel pressure tank near the instrument. Waste water is collected in a plastic container, also nearby. When the "Low Water Level" light on the instrument comes on, fill the tank with deionized water AND empty the waste tank. Never fill the water tank without also emptying the waste container! Add approximately 200 mL of bleach to the waste container prior to reconnecting it to the system so that waste water can be discarded down the sink as "decontaminated liquid waste." Never put any solution other than deionized water into the water tank. If the "Low Pressure" light on the instrument comes on, check the connections and seals on the stainless steel pressure tank.

- (2) Operation of COMMANDER incubator

Set the temperature to 45°C.

- (3) Operation of the QUANTIMATIC Plate Reader

NOTE: These Abbott instruments were approved for use with these test kits as part of FDA licensure of the kits.

- (a) After the final reaction has been stopped, place the tube rack(s) into the appropriate QUANTAMATIC carrier tray(s).
- (b) Place the carrier tray(s) onto the QUANTAMATIC to be automatically fed into the tube pick-up area.
- (c) On the instrument keypad choose RUN ASSAYS. Answer the prompts as shown in Table 1.

TABLE 1. QUANTAMATIC Parameters

Prompt	Response
Run which assay?	Assay # from TABLE 1
Lot #/Tech	Kit lot # and initials
Positive ID?	NO
Number of patients?	Number of patients
Tray 1 size - 20?	YES if tray size is 20 NO if tray size is 60
Is tray in back tray?	YES if tray is in the back track; NO if tray is in the front track
How many tubes in tray?	Total number of tubes
Enter pat no. ID	NO
List operator entries?	NO
Are trays ready?	YES if trays are ready to be read

D. Operation of Assay Procedure

- (1) Pipette 200 μ L of each control or test specimen into the wells of a reaction tray.
- (2) Carefully add one bead to each well containing a specimen or control.
- (3) Apply the cover seal. Gently tap the tray to cover the beads and remove any trapped air.
- (4) Incubate at 20–25°C (room temperature) overnight 16 hours.
- (5) Remove and discard the cover seal. Wash the beads in the tray using the QWIKWASH bead washer.
- (6) Immediately transfer beads to properly identified assay tubes.
- (7) Pipette 300 μ l of freshly prepared OPD substrate solution into two empty tubes (substrate blank) and then into each tube containing a bead. (NOTE: prime dispenser immediately prior to dispensing OPD substrate solution).
- (8) Cover and incubate are room temperature for 30 minutes.
- (9) Add 1 ml of 1 N sulfuric acid to each tube to stop color development.

- (10) Blank spectrophotometer with a substrate blank at 492 nm. Determine the absorbance of controls and test specimens at 492 nm within 2 hours after adding the acid.

E. Recording of Data

- (1) Quality Control Data

Multiple positive and negative controls are averaged by the reading instrument and are determined to be valid or invalid.

Raw data are transcribed manually from instrument readout sheet into a computerized database. Quality control of individual control values is maintained by the QUANTAMATIC, which will reject the test run if control values do not conform to specifications.

- (2) Analytical Results

Raw data are expressed as absorbance values and are transcribed manually from the instrument readout sheet into a computerized database.

F. Replacement and Periodic Maintenance of Key Components

- (1) Instruments are on service contract and except for the most basic daily maintenance are serviced by an Abbott technical representative.

Monitor and document incubator temperature, quality of water used in the QWIKWASH, refrigerator temperature, freezer temperature, and room temperature on a weekly basis

- (2) All micropipettors used in testing clinical specimens should be checked for calibration every 6 months. Pipettors that do not conform to specifications should be autoclaved and sent out for recalibration in accordance with the manufacturer's recommendations. Calibration records should be kept for each pipettor by serial number.

G. Calculations

- (1) Cutoff calculation is done by the reading instrument and by the data management software which use the following formula:

$$\text{Cutoff} = \text{NC}\bar{x} + 0.05,$$

where $\text{NC}\bar{x}$ is the mean absorbance value of the negative control.

- (2) Specimens with absorbance values greater than or equal to the Cutoff Value established with the negative control are to be considered reactive for HBsAg.

H. Procedure Notes

- (1) When dispensing beads, remove the cap from the bead bottle, attach the bead dispenser and dispense beads into wells of the reaction tray.
- (2) Do not splash liquid while tapping trays.
- (3) When washing beads, follow the directions provided with the washing apparatus.

9. REPORTABLE RANGE OF RESULTS

Final results are expressed qualitatively as positive or negative for the presence of HBsAg in the sample. No quantitative results are determined.

10. QUALITY CONTROL (QC) PROCEDURES

The method described in this protocol has been used extensively in the HRL for epidemiological studies. This method has proven to be accurate, precise, and reliable. The instrumentation used is state-of-art. Three negative controls, two positive controls, and one in-house control are included in each analytical run (a set of consecutive assays performed without interruption). The presence or absence of HBsAg is determined by comparing the absorbance value of the sample to the cutoff value. This cutoff value is calculated from the negative and positive control absorbance values as explained in the Calculations Section. Specimens with absorbances less than or equal to the cutoff value are considered non-reactive for HBsAg. Specimens that are reactive will be repeated in duplicate according to the package insert. An external positive anti-HBc control serum is purchased from Blackhawk Biosystems, Inc. and used in each testing run. When tested by using the Abbott Auszyme assay, the final HBsAg in-house control (IHC) reagent must generate a signal-to-cutoff ratio that falls within a specified range. This is developed by calculating a range of ± 3 standard deviations from the mean after performing multiple runs on separate days. This range is re-calculated for each lot purchased and is specified within the DMS. The precision of these procedures is as claimed for licensure and is maintained by the manufacturer under the authority of the FDA.

11. REMEDIAL ACTION IF CALIBRATION OR QC SYSTEMS FAIL TO MEET ACCEPTABLE CRITERIA

By definition, if controls do not conform to specifications, the testing is rejected. All samples are tested again. Data from nonqualifying test runs are not used.

Repeat the test for any violations of the following rules:

- All individual negative control values should be less than or equal to 0.100 and greater than or equal to -0.010 .
- Negative control values should be greater than or equal to 0.5 times the $NC_{\underline{x}}$ and less than or equal to 1.5 times the $NC_{\underline{x}}$.
- The P-N (difference between the mean of the positive and negative values) value must be 0.400 or greater.
- The cut-off is calculated by adding a factor of 0.5 to the $NC_{\underline{x}}$.

Some specimens that are reactive in the screening procedure may not be reactive on repeat testing. This phenomenon is highly dependent on technique used in running the test. The most common sources of such nonrepeatable reactives are:

- Improper bead washing
- Cross-contamination of nonreactive specimens caused by transfer of high-titer antigen specimen
- Contamination of the OPD substrate solution by oxidizing agents
- Contamination of the acid used as a stopping reagent
- Contamination of reaction tray well rim with anti-HBs conjugate reagent, or specimen

All highly sensitive immunoassay systems have a potential for nonspecific reactions, but high specificity of repeatably reactive specimens can be confirmed by neutralization tests. It is desirable to perform a

specificity analysis with an FDA-licensed neutralization confirmatory test prior to informing a donor/patient that he is an HBsAg carrier.

12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS

- A. The sample is restricted to human serum or plasma.
- B. No interfering substances have been identified.
- C. Although the association of infectivity and the presence of HBsAg is strong, presently available methods for HBsAg detection may not be sensitive enough to detect all potentially infectious units of blood or possible cases of hepatitis.
- D. Kit positive and negative controls must conform to the manufacturer's specifications in the kit insert literature.
- E. In-house controls must have a signal-to-cutoff ratio that falls within the range determined for each individual lot.

13. REFERENCE RANGES (NORMAL VALUES)

A normal value for HBsAg should be negative.

14. CRITICAL CALL RESULTS (PANIC VALUES)

Not applicable.

15. SPECIMEN STORAGE AND HANDLING DURING TESTING

Specimens may remain at 20–25°C during preparation and testing for 4 hours.

16. ALTERNATIVE METHODS FOR PERFORMING TEST OR STORING SPECIMENS IF TEST SYSTEM FAILS

Other FDA-licensed tests for total HBsAg may be substituted but must be accompanied by validation data to show substantial equivalence with these assays. Test methods may not be substituted without approval from NCHS.

17. TEST RESULT REPORTING SYSTEM; PROTOCOL FOR REPORTING CRITICAL CALLS (IF APPLICABLE)

Not applicable.

18. TRANSFER OR REFERRAL OF SPECIMENS; PROCEDURES FOR SPECIMEN ACCOUNTABILITY AND TRACKING

Test results are documented through the lab management database (Section 3). Generally, studies conducted in the HRL are sponsored by a CDC epidemiologist who communicates the findings to other participants in the study. Final reports may be electronic or in printed form. All electronically held data are backed up routinely.

Specimens in long-term storage specimens are arranged by study group. The storage location of each sample is listed with the test data.

19. SUMMARY STATISTICS AND GRAPHS

Qualitative assays are qualitative assays with a positive, negative or borderline/indeterminate result. The absorbance or reactivity values of specimens are compared with a cutoff value that is a ratio of the negative control mean and the positive control mean. Since the controls are read as cutoff values, plots of these values are not generated for quality control purposes.

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