Laboratory Procedure Manual

Analyte: **Hepatitis B Core Antibody**

Matrix: **Serum**

Method: **ORTHO HBc ELISA Test System 480 Test Kits**

Method No.:

Revised:

as performed by: *Hepatitis Branch*
Division of Viral Hepatitis
National Center for Infectious Diseases

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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 NHANES 2001–2002 data.

A tabular list of the released analytes follows:

Lab Number	Analyte	SAS Label
l02_b	LBXHBC	Hepatitis B core antibody

1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

ORTHO HBc ELISA Test System is a qualitative enzyme-linked immunosorbent assay for the detection of total antibody to hepatitis B virus core antigen (anti-HBc) in human serum or plasma indicated for the screening of blood and blood products intended for transfusion and as an aid in the diagnosis of ongoing or previous hepatitis B virus (HBV) infection.

Anti-HBc appears in virtually all individuals infected with HBV and is an accurate serological marker of recent and past infection (1, 2). During the acute phase of HBV infection, anti-HBc appears shortly after the appearance of hepatitis B surface antigen (HBsAg) and persists following HBsAg clearance (3). In those cases where HBsAg has cleared and the appearance of anti-HBs is delayed, anti-HBc may be the only serological marker of recent HBV infection (4). Anti-HBc is found in virtually all patients with chronic hepatitis B (5).

Enzyme-linked immunosorbent assay (ELISA) procedures provide a means for routinely detecting antibodies to specific antigens (6, 7). This FDA-licensed method is commercially obtained in kit form. The literature and instructions with each kit constitute the standard operating procedure (SOP) for the method.

2. SAFETY PRECAUTIONS

Test kits for the ELISA 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 kit components as though they are capable of transmitting disease. Consider all serum specimens for analysis potentially positive for infectious agents including HIV, HBV and the hepatitis C virus (HCV) . Observe universal precautions; wear protective gloves, eyewear, and lab coat during all steps of this method because of both infectious and chemical contamination hazards. Place all plastic and glassware contaminated with serum in a plastic autoclave bag for disposal. We recommend Biosafety Level 2 containment procedures as described in CDC/NIH publication #88-8395 be used by those handling test specimens and kit reagents (8).

Material safety data sheets (MSDSs) for sodium azide, sulfuric acid, hydrochloric acid, o-phenylenediamine, and sodium hypochlorite are available through the National Center for Infectious Diseases (NCID) computer network. Risk is minimal due to the small quantity of chemicals, the safe packaging, and the limited handling 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.

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 µL 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. PREPARATION OF REAGENTS, CALIBRATORS (STANDARDS), CONTROLS, AND ALL OTHER MATERIALS; EQUIPMENT AND INSTRUMENTATION

   A. Instrumentation

      (1) Multichannel aspirator-washer device capable of dispensing and aspirating 300 µl to 800 µl per well.

      (2) Dual wavelength microwell reader capable of reading at 490 nm or 492 nm with a reference filter of 620 nm or 630 nm. Linearity of the microwell reader must range from at least 0 to 2.5 absorbance units. Adjustable multichannel micropipette capable of delivering 50 µl and 200 µl with at least ± 5% accuracy or equivalent reagent dispenser.

(3) Fixed or adjustable single-channel micropipette capable of delivering 10 µl with at least ± 10% accuracy or equivalent sample dilutor.

B. Materials

(1) Deionized water (Continental Water Systems, Inc., San Antonio, TX).

(2) 5 µl and 250 µl disposable pipette tips or equivalent.

(3) Multichannel micropipette reservoir or equivalent reagent container.

(4) Protective gloves, Tronex or Flexam, small/medium/large (Best Manufacturing, Menlo Park, GA).

(5) 2 mL cryovials, cat. no. 5000-0020 (Nalge Company, Inc., Rochester, NY).

(6) Cryovial boxes, cat. no. 5026-0909 (Nalge Company, Inc., Rochester, NY).

(7) 1.5 mL microtubes (Marsh Biomedical Products, Rochester, NY).

(8) 50 mL-polypropylene tubes (Corning Glass Works, Corning, NY).

(9) ORTHO HBc ELISA Test System 480 Test Kit (Product Code 933245, Ortho Diagnostic Systems, Raritan, NJ).

(10) 4N sulfuric acid (H2SO4) (Product Code 933040, Ortho Diagnostic Systems, Raritan, NJ).

(11) 20X Wash Buffer Concentrate (Product Code 933730, Ortho Diagnostic Systems, Raritan, NJ). Phosphate buffer with sodium chloride and detergent. Preservative: 2% 2-chloroacetamide.

C. Reagents

ORTHO HBc ELISA Test System 480 Test Kits contain the following reagents; prepared by the manufacturer. Volumes listed are for 480 tests.

(1) Hepatitis B Virus Core Antigen (HBcAg) (Recombinant)-Coated Microwell Plates
5 Plates. Each plate has 8 strips of 12 wells in each holder.

(2) Antibody Conjugate (Murine Monoclonal)
1 bottle (125 mL). Mixture of anti-human IgG and anti-human IgM conjugated to horseradish peroxidase with protein stabilizers. Preservative: 0.02% thimerosal.

(3) Specimen diluent
1 bottle (150 mL). Phosphate-buffered saline with protein stabilizers and detergents. Preservative: 0.02% thimerosal.

(4) OPD Tablets
30 Tablets. *o*-phenylenediamine•2HCl.

(5) Substrate buffer
1 bottle (190 mL). Citrate-phosphate buffer with 0.02% hydrogen peroxide. Preservative: 0.01% thimerosal.

(6) Positive control (Human)
1 vial (1.0 mL). Source: Human serum or plasma containing anti-HBc and non-reactive for HBsAg and antibody to human immunodeficiency virus type 1 (HIV-1) and type 2 (HIV-2). Preservative: 0.02% thimerosal.

(7) Negative control (Human)
2 vials (1.0 mL each). Source: Human serum non-reactive for anti-HBc, HBsAg and antibody to human immunodeficiency virus type 1 (HIV-2) and type 2 (HIV-2). Preservative: 0.02% thimerosal.

(8) Plate sealers
21, disposable.

D. Reagent Preparation

(1) Preparation of Wash Buffer (1X): Mix 50 ml of 20X Wash Buffer Concentrate with 950 ml of distilled or deionized water. Wash Buffer (1X) is stable for 30 days when stored at room temperature. For longer storage (up to 60 days), keep at 2 to 80 C. Record the date the Wash Buffer (1X) is prepared and the expiration date on the container. Discard Wash Buffer (1X) if visibly contaminated.

(2) Preparation of Substrate Solution: Clean glass or plastic vessels must be used. Prior to the end of the second incubation, transfer a sufficient amount of Substrate Buffer to a container and protect the contents from light. Completely dissolve the appropriate number of OPD tables in Substrate Buffer prior to use.

Each Microwell plate requires at least 20 mL of Substrate Solution. More Substrate Solution may be needed depending upon the reagent dispenser used. Below are guidelines for general use.

Number of Wells	Number of Plates	Number of OPD Tablets	Substrate Buffer (mL)
48	0.5	1	12
96	1	2	24
144	1.5	3	36
192	2	4	48
240	2.5	5	60
288	3	6	72

The Substrate Solution is stable for 60 minutes after the addition of OPD tablets when held at room temperature in the dark and should be colorless to very pale yellow when used.

7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

A. Calibration Curve

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

B. Verification

Not Applicable

8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

A. Preliminaries

(1) Prior to the beginning of the procedure, bring kit components to room temperature (15–30°C). Invert liquid reagents gently several times, but avoid foaming. Check the incubator temperature; maintain at 37°C ± 1°C.

(2) Determine the total number of wells needed for the assay. In additional to specimens, one substrate blank, three negative controls and two positive controls will be included on each plate or partial plate. If the entire strip is not needed, an appropriate number of wells can be broken off. Unused wells should be stored at 2–8°C in the supplied foil pouch, tightly sealed with desiccant and used within 14 days of opening the foil pouch. Record the date the pouch is opened and the expiration date of the unused wells on the pouch.

Performing the test on less than a full plate is permitted as long as the following conditions are met:

- Microwell strips from different plates can be mixed to assemble full or partial plates as long as they are from the same lot, within the open pouch expiration date and have come from plates that have previously demonstrated proper response to kit controls.

- When assembling a plate that contains strips from newly opened, previously untested plate, one of these strips should be placed at the beginning of the plate and received the full complement of kit controls.

  CAUTION: Handle microwell strips with care. Do not touch the bottom exterior surface of the wells.

(3) Once the assay has been started, complete all subsequent steps without interruption and within the recommended time limits.

B. Sample Preparation

(1) Allow the serum specimens to come to 20–25°C. Serum and plasma samples may stratify when frozen or stored at 4–8°C for extended periods. Mix the specimens gently before testing.

(2) Assemble the microwell strips into the microwell strip holder, if necessary. Microwell strips must be level in the microwell strip holder. For incomplete plates, add black or uncoated microwell strips.

(3) Prepare a record (plate map) identifying the placement of the controls and specimens in the microwells.

(4) Arrange the assay control wells so that well 1A is the substrate blank. From well 1A, arrange all controls in a horizontal or vertical configuration as follows: Configuration is dependent upon software.

Well 1A	Substrate blank
	Negative Control
	Negative Control
	Negative Control
	Positive Control
	Positive Control

C. Instrument Setup

(1) Operation of the Ortho AutoWash

The Ortho AutoWash is a semi-automated instrument that is used to wash microtiter plates between reagent steps. The wash solution is prepared as per section 6.D.1.

(a) Turn on the AutoWash 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) Place the microwell plate on the plate support.

(c) Using the keypad the user selects the preprogrammed wash cycle for Ortho HBc.

(d) Press the Start button.

(e) After the wash cycle is over remove the plate.

(f) Set the AutoWash to Soak.

(g) Thermolyne incubator

(h) Set and verify the temperature of the incubator at 37°C.

(i) Operation of the Ortho AutoReader II

(j) After the final reaction has been stopped, place the microwell plate on the carrier plate.

(k) Using the computer keyboard select the preprogrammed Sanguin assay to control the reading of the microwell plate with the AutoReader II by using the cursor keys to select GENERATE.

(l) Select PLATE followed by GENERATE/EDIT A PLATE and press the ENTER key.

(m) From the Assay column choose HCV 3.0 and enter a plate number and press ENTER

(n) From the computer keyboard enter the information about the assay controls and sample ID numbers into the proper positions on the microwell template on the computer screen and press END

(o) Select RETURN TO MASTER MENU.

(p) Go to the MASTER MENU and select READ A PLATE.

(q) The Ortho AutoReader will automatically read the plate, and print the absorbance value for each well to a printer.

D. Operation of the Assay Procedure

(1) Pipette 200 µL of Specimen diluent to all wells of the microtiter plate except well 1A.

(2) Pipette 10 µL of controls or serum samples into the appropriate wells.

(3) Apply cover seal. Incubate at $37^{o}C \pm 1^{o}C$ for 1-hour ± 5 minutes.

(4) Place the microtiter plate on the AutoWash and wash all the wells five times with Wash Buffer (1X).

(5) Add 200 µL of Antibody Conjugate to all wells except 1A.

(6) Apply cover seal. Incubate at $37^{o}C \pm 1^{o}C$ for 1-hour ± 5 minutes.

(7) Prepare sufficient Substrate Solution to fill the control and test wells. Allow time for the OPD tablets to dissolve completely.

(8) Place the microtiter plate on the AutoWash and wash all the wells five times with Wash Buffer (1X).

(9) Add 200 µL of Substrate Solution to all the wells including 1A.

(10) Apply cover seal. Incubate at room temperature for 30 minutes ± 1 minute in the dark.

(11) Add 50 µL of 4N sulfuric acid (H_2SO_4) to all wells including 1A.

(12) Read the reaction at 492 nm in the Ortho AutoReader II spectrophotometer.

(13) Retest positive samples in duplicate using this procedure.

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 the instrument readout sheet into a computerized database.

(2) Analytical Results

For ELISA, raw data are expressed as absorbance value. Raw data 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 Ortho Clinical Diagnostics technical representative.

(2) The following procedures are monitored and documented on a weekly basis:

- incubator temperature
- operation of the Ortho AutoWash
- refrigerator temperature
- freezer temperature
- room temperature

(3) All micro-pipettors 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 re-calibration in accordance with manufacturer's recommendations. Calibration records should be kept for each pipette by serial number.

G. Calculations

(1) The cutoff calculation is done by the reading instrument and by the data management software which uses the following formula:

$$\text{Cutoff} = \text{NC}\underline{x} + 0.400$$

(2) Calculate the negative control mean absorbance (NC$\underline{x}$) by determining the mean of the negative controls.

H. Replacement and Periodic Maintenance of Key Components

(1) Replacement:

When the internal printer is out of paper, the Check Printer pop-up screen or Printer Error screen displays, prompting you to check the printer paper supply and replenish if necessary.

(2) Maintenance:

Periodic maintenance of the RIBA Processor System involves both end-of-run maintenance procedures as well as monthly maintenance procedures. Both should be recorded in Maintenance Log.

End-of-Run Maintenance:

- Clean the instrument
- Dispose of the reagent container fluids
- Clean reagent containers
- Dispose of reaction vessels
- Check waste container – empty if necessary

Monthly Maintenance:

- Clean reaction chamber bowl
- Clean detection windows
- Clean air filter
- Clean outside of probe

I. Special Procedure Notes

None

9. REPORTABLE RANGE OF RESULTS

A normal value for anti-HBc should be negative. Final reports express results qualitatively as positive or negative for the presence of anti-HBc antibody in the sample. No quantitative results are determined. All samples testing initially positive are retested in duplicate.

10. QUALITY CONTROL (QC) PROCEDURES

The method described in this protocol has been used for several years in the HRL for epidemiologic studies. This method has proven to be accurate, precise, and reliable. The instrumentation used is state-of-art.

This quality control system uses bench quality control samples. Positive and negative controls are included with kits. An external positive anti-HBc control serum is purchased from Blackhawk Biosystems,Inc. and used in each testing run. When tested by using the anti-HBc ELISA, the final anti-HBc 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.

Three negative controls, three 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 anti-HBc 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 absorbance greater than or equal to the cutoff value are considered reactive for anti-HBc.
For the run to be valid, the difference between the mean absorbance of the positive and negative (P-N) must be 0.400 or greater. If not, poor technique or reagent deterioration should be suspected, and the run should be repeated.

Individual negative control values must meet the following acceptance criteria:

- less than or equal to 0.350, and greater than or equal to –0.005.
- If one of the 3 negative control values is outside either of these limits, recalculate the negative control mean based on the other two acceptable values. The plate is invalid and the run must be repeated if two or more of the control values are outside of the limits.

Individual positive control values must meet the following acceptance criteria:

- greater than or equal to 0.800
- within the linear range of microwell reader
- must not differ by more than 0.500

Precision of these procedures is as claimed for licensure and is maintained by the manufacturer under the authority of the FDA.

This method generates coefficients of variation (CVs) of 5–10% within runs and 8–15% between runs in the HRL.

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

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

B. If one negative control value fails to meet either of the acceptance criteria in the quality control procedures, it must be excluded and the $NC\underline{x}$ recalculated. All remaining individual control values must meet the criteria or the run should be repeated.

C. An assay run is considered valid with respect to substrate blank if the blank has an absorbance value greater than or equal to –0.020 and less than or equal to 0.050. The user must determine assay validity due to substrate blank.

D. If the substrate blank falls outside the acceptable range, the preparation of the substrate is in question and the alternate blank may be used. If it is unacceptable, the run must be repeated.

12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS

A. The sample must be human serum or plasma.

B. No interfering substances are identified.

C. The test procedure is limited to the detection of anti-HBc in serum or plasma. Currently available methods for anti-HBc detection may not detect all potentially infectious units of blood or possible cases of hepatitis B. As with any diagnostic test, false reactive results may be obtained.

13. REFERENCE RANGES (NORMAL VALUES)

Normal human serum is negative for hepatitis B core antigen.

Samples generating values ±10% of the cutoff are repeated.

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 anti-HBc may be substituted but must be accompanied by validation data to show substantial equivalence with these assays. Substitution of test methods may not be done without approval from the NCHS.

Alternate storage is not recommended.

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, a CDC epidemiologist 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 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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