

Laboratory Procedure Manual

Analyte: **Hepatitis A Antibody**

Matrix: **Serum**

Method: **HAVAB-EIA Test Kits**

Method No.:

Revised:

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

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March 2008

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 items listed in the following table:

Data File Name	Variable Name	SAS Label
HEPHA_D	LBXHA	Hepatitis A antibody(anti-HAV)

1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

A qualitative determination of total antibody to hepatitis A virus (anti-HAV) contained in human serum or plasma is measured by using solid-phase competitive enzyme immunoassay (EIA) (1-3). A test sample is mixed with detection-phase reagent in a reaction well. The detection-phase reagent consists of anti-HAV conjugated with peroxidase (anti-HAV/PO). The sample-conjugate mixture is incubated with a bead coated with HAV antigen. Any anti-HAV in the test sample competes with the conjugate for HAV epitopes present on the bead. Thus, at the end of the incubation period, the amount of conjugate immunochemically bound to the bead will be inversely proportional to the concentration of anti-HAV in the sample. The beads are washed to remove any unbound material. The beads are then incubated with a hydrogen peroxide/o-phenylenediamine (H₂O₂/OPD) chromogenic substrate solution. The reaction of substrate solution with peroxidase yields a yellow-orange color. The reaction is stopped by the addition of 1-N sulfuric acid. The intensity of the color generated is measured spectrophotometrically at 492 nm. The instruments used to measure the test results are equipped with software that calculates a cutoff value. The cutoff calculation is based upon values obtained from control reagents included with each testing series. Results are expressed as "positive" or "negative" for anti-HAV.

This is a FDA-licensed method commercially obtained in kit form. The literature and instructions with each kit constitute the standard operating procedure (SOP) for the method. Its diagnostic utility lies in the determination of whether an individual has been infected with hepatitis A virus, for which anti-HAV is a marker. It can also be used to assess the efficacy of HAV vaccines.

The presence of anti-HAV in human serum is indicative of past or present infection with hepatitis A virus. Virus specific anti-HAV IgM is the most reliable marker for determining the acute stage of disease. Total anti-HAV may persist for years after recovery as anti-HAV IgG. The test for total anti-HAV is primarily used to determine previous exposure to Hepatitis A virus. The results of these tests may be used to assess immune status (5, 6) for epidemiological studies (7-12).

2. SAFETY PRECAUTIONS:

EIA test kits for anti-HAV 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.

Consider all serum specimens for analysis 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 (13).

Material safety data sheets (MSDSs) for sulfuric acid, hydrochloric acid o-phenylenediamine, and sodium hypochlorite 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 instrument readout sheets into a computerized database. This database was custom-designed for the management of CDC Hepatitis Reference Laboratory (HRL) test results. It functions within PARADOX software (Borland Co., Scott's Valley, CA). Test values are compared with a cutoff value calculated from controls. Results are expressed as "positive" or "negative" for anti-HAV. 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.

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- b. Finished data are reviewed by the supervisor. After each NHANES stand is completed (i.e., when all clinical evaluations and analyses from each mobile survey site are complete) the supervisors will be transmit the results to the NCHS mainframe computer along with the other NHANES 99+ data.
- c. Files stored on the LAN or CDC mainframe are automatically backed up nightly to tape by CDC Data Center staff.
- d. Documentation for data system maintenance is contained in hard 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" (S. Lambert, 5/92).
- 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 by using regular red-top or serum-separator Vacutainers.
- d. Required sample volume is 10 μ L for the assay and 1.0 mL will permit repeat analysis.
- e. Specimens may be stored in glass or plastic vials, as long as the vials are tightly sealed 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 Nalge cryovials or an equivalent vial.
- h. Serum is best stored frozen. However, freeze/thaw cycles should be minimized. Specimens may be stored at 4-8 °C for up to 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. Do not use heat-inactivated specimens.
- l. Specimens containing sodium azide may give false-positive reactions and should not be tested.

**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**

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- (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) HAVAB-EIA solid phase EIA kit, 100- or 1000-test sizes, cat. no. 789524 (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) 1.0 N sulfuric acid, cat. no. 7212 (Baxter).
- (6) Pipet tips, cat. nos. RT20 & RT200 (Rainin Instrument Co.).
- (7) Protective gloves, Tronex or Flexam, small/medium/large (Best Manufacturing, Menlo Park, GA).
- (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. Each kit contains the following:
 - (a) Hepatitis A virus (human) coated beads
1 bottle (100 beads).
 - (b) Antibody to hepatitis A virus (human)
1 vial (20 mL). Peroxidase (horseradish) conjugate. Minimum concentration: 0.05 μ g/mL in buffer. Antimicrobial agents.
 - (c) Negative control
1 vial (0.45 mL). Recalcified human plasma nonreactive for anti-HAV, HBsAg, and anti-HIV-1. Antimicrobial agents.
 - (d) Positive control
1 vial (0.30 mL). Recalcified human plasma reactive for anti-HAV, nonreactive for HBsAg and anti-HIV-1. Titer 1:200 \pm 2 \log_2 dilutions. Antimicrobial agents.
 - (e) Diluent for o-phenylenediamine•2HCL (OPD)
1 bottle (55 mL). Citrate-phosphate buffer containing 0.02% (v/v) hydrogen peroxide.

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- (f) o-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 prepares 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**

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

Positive and negative control reagents are supplied with each test kit. The assay cutoff value is automatically calculated from values obtained from these controls by the instrument.

- (1) An "in-house control" (IHC) is included with each testing series. The IHC reagent is produced by the HRL according to an SOP entitled "In-House Control: T anti-HAV" (S. Lambert, 8/92). (See Section 6.e.).

e. **Preparation of Quality Control Material**

- (1) Kit positive and negative controls are prepared and quality controlled by the manufacturer.
(2) In-house controls are prepared according to HRL specifications.

A pool of anti-HAV positive serum is calibrated by serial dilution in a controlled-process serum-based diluent. *When tested by the anti-HAV EIA, the final anti-HAV IHC reagent must consistently generate a signal-to-cutoff ratio of 0.5 to 1.0.*

Dilute the previously characterized anti-HAV serum pool 1:25, 1:50, 1:100, 1:400 and 1:800 in a defibrinated, dialyzed-filtered, plasma pool diluent that is nonreactive for hepatitis A, B, and C. Test each dilution plus diluent at least in duplicate in HAVAB-EIA. Pick a dilution that yields a signal-to-cutoff value between 0.6 and 0.9. Prepare 8-9 mL of the chosen dilution. Test at least in duplicate in HAVAB-EIA to confirm that the signal-to-cutoff value is between 0.6 and 0.9. Aliquot the control material into labeled vials with 40-50 µL per vial. Store at -20 °C. Thaw each in-house control as needed, use once, and discard.

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**

- (1) The instrument used to read assay results (Section 6.e.4) 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.
(2) Test results that fall within $\pm 10\%$ of the calculated cutoff value are reanalyzed.

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- (3) If two negative control values fall outside 0.7-1.3 times mean, repeat the test.
- (4) Calculate the difference between the negative control and positive control mean absorbance values (N-P value) as a measure of the validity of the test. For the run to be valid, the N-P value should be at least 0.400. If it is not, repeat the run. If the N-P value is consistently less than 0.400, deterioration of reagents may be the cause.

8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

a. Preliminaries

- (1) Reagents are used per kit of 100 or 1000 tests. 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. Adjust the incubator to 40 ±1 °C.
- (3) Assay the negative, positive, and in-house controls in triplicate with each run of specimens.
- (4) Ensure that all reaction trays are subjected to the same process and incubation times.
- (5) Once the assay has been started, complete all subsequent steps without interruption and within the recommended time limits.
- (6) After each step, visually verify the presence of solution and bead in each well.

b. Sample Preparation

- (1) Bring serum specimens to 20-25 °C. Serum and plasma samples may stratify when frozen or stored at 4-8 °C for extended periods. Mix them gently before testing.
- (2) Identify 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.

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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 empties 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) COMMANDER incubators: Set at 40 °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 # & 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 track?	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 10 µL of controls and serum samples into designated wells of a reaction tray. Run order is three negative controls, followed by two positive controls and samples.
- (2) Pipette 200 µL peroxidase-conjugated anti-HAV antibody into each well containing a control or sample.
- (3) Add one HAV antigen-coated bead to each well.

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- (4) Apply the cover seal. Gently tap the tray to cover the beads and remove any trapped air.
- (5) Incubate at 20-25 °C for 18-24 hours.
- (6) Wash the beads in the tray using the QWIKWASH bead washer.
- (7) Transfer the beads to 10- x 75-mm tubes. Pipette 300 µL of substrate solution into two blank tubes and then into each tube containing a bead. Following 30-min incubation, stop the chromogenic reaction by adding sulfuric acid. Measure the intensity of the color generated using the QUANTAMATIC spectrophotometer.
- (9) Measure the absorbance of controls and 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 are checked for calibration every 6 months. Pipettors that do not conform to specifications are autoclaved and sent out for recalibration in accordance with the manufacturer's recommendations. Calibration records are kept for each pipettor by serial number.

g. **Calculations**

Cutoff calculation is done by the reading instrument and by the data management software.

- (1) Calculate the negative control mean absorbance (NC) by dividing the total absorbance by the total number of negative controls.

All negative control values should fall within 0.7-1.3 times the mean. If one value is outside this range discard this value and recalculate NC. If two values are outside the range, repeat the test. If more than an occasional value falls outside this range, technique problems should be suspected.

- (2) Calculate the positive control mean absorbance (PC) by dividing the total absorbance by the total number of positive controls.

- (3) Determine the cutoff value by dividing the sum of NC and PC by 2.

- (4) Specimens with values that are within $\pm 10\%$ of the cutoff value are reanalyzed.

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- (5) Calculate the N-P value by subtracting PC from NC. The N-P value should be 0.400 or greater. If not, technique problems or reagent deterioration should be suspected and the run repeated.
- (6) An in-house control (IHC) is included with each testing series. The IHC reagent is produced by the HRL according to a SOP entitled "In-House Control: T anti-HAV" (S. Lambert, 8/92). A pool of anti-HAV positive serum is calibrated by serial dilution in a controlled process serum-based diluent. *When tested using the anti-HAV EIA the final anti-HAV IHC reagent must consistently generate a signal-to-cutoff ratio of 0.5 to 1.0.*
- (7) Specimens with absorbance less than or equal to the cutoff value are considered reactive for anti-HAV. Specimens that are reactive are considered positive by the criteria of the test kit.

h. Special 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.
- (4) When transferring beads from wells to assay tubes, align an inverted carton of tubes over their respective wells in the reaction tray. Press the tubes tightly over the wells and invert the tray and tubes together so that the beads fall into the corresponding tubes. Blot excess water from top.
- (5) Avoid strong light during color development.
- (6) Dispense acid in the same sequence as the OPD substrate solution.
- (7) Do not allow acid or OPD substrate solution to contact metal.
- (8) Remove air bubbles prior to reading absorbance.
- (9) Visually inspect blank tubes and discard them if they have a yellow-orange color. If both blank tubes are contaminated repeat the entire run.

9. REPORTABLE RANGE OF RESULTS

Final results are expressed qualitatively as positive or negative for the presence of anti-HAV antibody in the sample. No quantitative results are determined.

10. QUALITY CONTROL (QC) PROCEDURES

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

This quality control system uses bench quality control samples. Positive and negative controls are included with kits. One in-house control is a pool of anti-HAV positive serum calibrated by serial dilution in a controlled-process serum-based diluent. When tested by the anti-HAV EIA, the final anti-HAV IHC reagent must consistently generate a signal-to-cutoff ratio of 0.5 to 1.0.

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 anti-HAV is determined by comparing the absorbance value of the sample to the cutoff value. This

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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 reactive for anti-HAV. Specimens that are reactive are considered positive by the criteria of the test kit.

The difference between the negative control and positive control mean absorbance values (N-P) is calculated as a measure of the validity of the test. For the run to be valid, the N-P value should be at least 0.400 or greater. Samples with test results that fall within $\pm 10\%$ of the calculated cutoff value are reanalyzed.

The 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 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. If controls do not conform to specifications, reject the results and reanalyze all samples. Do not use data from nonqualifying test runs.
- b. Samples with test results that fall within $\pm 10\%$ of the calculated cutoff value are reanalyzed.
- c. If two negative control values fall outside 0.7-1.3 times the mean, repeat the test.
- d. Calculate the difference between the negative control and positive control mean absorbance values (N-P) as a measure of the validity of the test. For the run to be valid, the N-P value should be at least 0.400. If it is not, repeat the run. If the N-P value is consistently less than 0.400, deterioration of reagents might be the cause.

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. Multiple/freeze thaw procedures should be avoided.
- d. The HAV EIA test is limited to the qualitative detection of anti-HAV in human serum or plasma.

13. REFERENCE RANGES (NORMAL VALUES)

A normal human serum should be negative for hepatitis A antibodies.

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. ALTERNATE METHODS FOR PERFORMING TEST OR STORING SPECIMENS IF TEST SYSTEM FAILS

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Other FDA-licensed tests for total anti-HAV 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.

Alternative methods of storage are not recommended. In case of system failure, samples should be refrigerated at 4-8 °C for no more than 5 days. For longer periods, the specimens should be stored at - 20 °C until the system is functioning properly.

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 are arranged by study group. The storage location of each sample is listed with the test data.

19. Summary Statistics and QC 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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ACKNOWLEDGMENTS

We gratefully acknowledge the contributions of Miriam Alter, Joni Kaluba, Stephen Lambert, Brian Mahy, Ph.D., Harold Margolis, M.D., Linda Moyer and Mar Yi Than who assisted in developing the methodology, performing the analysis for the antibody to hepatitis A in the NHANES III study, and preparing the manuscript for this chapter.