

Laboratory Procedure Manual

<i>Analyte:</i>	Hepatitis C Antibody/Hepatitis C Confirmatory Test
<i>Matrix:</i>	Serum
<i>Method:</i>	Ortho HCV Version 3.0 ELISA Test Kit and Chiron RIBA HCV Version 3.0 Strip Immunoblot Assay Kit
<i>As performed by:</i>	<i>Hepatitis Branch Division of Viral Hepatitis National Center for Infectious Diseases</i>
<i>Contact:</i>	<i>Dr. Wendi Kuhnert</i>

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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:

Lab	Analyte	SAS Label	Description
HEPC_E	LBDHCV	Hepatitis C antibody	Hepatitis C antibody confirmed

Screening Enzyme Immunoassay

1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

Qualitative determination of the human antibody directed against hepatitis C virus (anti-HCV) in human serum or plasma is measured using direct solid-phase enzyme immunoassay. Human serum or plasma is diluted in specimen diluent and incubated on a microwell coated with recombinant HCV antigen. Following a 1-hour incubation, the plate is washed to remove unbound material. A peroxidase-conjugated antibody directed against human IgG is added to each well on the microwell plate. Following a 60-min incubation, the wells are washed again to remove unbound material. A peroxidase-specific chromogenic substrate solution is added to each well. The substrate solution consists of hydrogen peroxide and o-phenylenediamine (OPD) in a citrate buffer. Following a 30-min incubation at 20-25°C, 1 N sulfuric acid is added to stop the enzyme-substrate reaction.

Anti-HCV antibody will bind to the HCV antigen in the microwell. Subsequently, the conjugate binds to that antibody. The reaction of the conjugate with the substrate solution results in the generation of an orange color. Absence of color indicates the absence of anti-HCV in the sample. The intensity of the color generated is measured spectrophotometrically at 492 nm. A cutoff value is calculated based upon values obtained from control reagents included with each testing series. Results are expressed as "positive" or "negative" for anti-HCV. Positive specimens are repeated in duplicate according to the same procedure. Repeatedly positive specimens are tested supplementally using the RIBA Processor System (Chiron Corporation, Inc.). While the Chiron RIBA 3.0 Strip Immunoblot Assay (Chiron Corporation, Inc.) is a licensed technology, the RIBA Processor System is not, but is in use by the HRL under an Investigational New Drug (IND) agreement.

This is an FDA-licensed method commercially obtained in kit form (1-3). The literature and instructions in each kit constitute the standard operating procedure (SOP) for the method. Its diagnostic utility lies in its capacity to determine whether an individual has been infected with hepatitis C. The presence of these antibodies indicates that the individual has been infected with HCV.

2. SAFETY PRECAUTIONS

Test kits for anti-HCV 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 and the hepatitis B virus. 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 (4).

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

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the controls. Results are expressed as "positive" or "negative" for anti-HCV. 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 supervisor. After each NHANES stand is completed (i.e., when all clinical evaluations and analyses from each mobile survey site are complete), the supervisor will transmit the results to SQL Server along with other NHANES IV data.
- C. Files stored on the CDC LAN are automatically backed up nightly to tape by CDC Data Center staff.
- D. Documentation for data system maintenance is contained in printed copies of data records for 2 years. .
SPECIMEN COLLECTION, STORAGE, AND HANDLING PROCEDURES; CRITERIA FOR SPECIMEN REJECTION
- E. Specimens submitted for testing are handled according to the HRL SOP entitled "Sample Handling" (W. Kuhnert, 3/03).
- F. No special instructions such as fasting or special diets are required. Diurnal variation is not a major consideration.
- G. Specimens may be serum, recalcified plasma, or plasma. Serum specimens may be collected using regular red-top or serum-separator Vacutainers.
- H. Required sample volume is 50 µL for the assay; 1.0 mL will permit repeat analyses as well.
- I. Specimens may be stored in glass or plastic vials, as long as vials are tightly sealed to prevent desiccation of the sample.
- J. Serum or plasma samples are collected aseptically to minimize hemolysis and bacterial contamination.
- K. Samples are stored in labeled 2 mL Nalge cryovials or equivalent.
- L. 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.
- M. 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.
- N. Specimens are rejected if contaminated, hemolyzed, or stored improperly. However, rejection is done only after consultation with NCHS.
- O. Avoid multiple freeze/thaw cycles.
- P. Do not use heat-activated specimens.
- Q. Performance has not been established for cadaver specimens or body fluids other than serum or plasma (such as urine, saliva or pleural fluid.)

4. PROCEDURES FOR MICROSCOPIC EXAMINATIONS; CRITERIA FOR REJECTION OF INADEQUATELY PREPARED SLIDES

Not applicable for this procedure.

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

A. Instrumentation

- (1) Ortho AutoWash 96, model 936380, Serial No. 71 852 (Ortho Diagnostic Systems, Raritan, NJ).
- (2) Thermolyne, Type 37900 Culture Incubator, model 137925, set at 37°C (Barnstead, Dubuque, IA).
- (4) Ortho AutoReader II, model 935060, Serial No. 61195 (Ortho Diagnostic Systems, Raritan, NJ).
- (5) Ortho Sanguin MEDUSA 2000 software, version 3.50 r2 (Ortho Diagnostic Systems, Raritan, NJ).

Note: The Ortho instruments cited above are approved for use with these test kits as part of FDA licensure of the kits.

- (6) Gilson Pipetman micropipettors, 10- and 200 µL sizes (Rainin Instrument Co., Woburn, MA).

B. Materials

- (1) Reaction trays, Costar cat.no. 4870 (VWR Scientific, Bridgeport, CT).
- (2) Cover seals provided as part of the test kit (Abbott Laboratories).
- (3) Deionized water (Continental Water Systems, Inc., San Antonio, TX).
- (4) Pipet tips, cat. nos. RT20 & RT200 (Rainin Instrument Co.).
- (5) Protective gloves, Tronex or Flexam, small/medium/large (Best Manufacturing, Menlo Park, GA).
- (6) 2 mL cryovials, cat. no. 5000-0020 (Nalge Company, Inc., Rochester, NY).
- (7) Cryovial boxes, cat. no. 5026-0909 (Nalge Company, Inc.).
- (8) 1.5 mL microtubes (Marsh Biomedical Products, Rochester, NY).
- (9) 50 mL-polypropylene tubes (Corning Glass Works, Corning, NY).
- (10) 5.25% sodium hypochlorite (household bleach) (any vendor).
- (11) Virotrol-I, cat. no. 00100 (Blackhawk Biosystems, Inc., San Ramon, CA)
- (12) Ortho HCV Version 3.0 ELISA Test System, Hepatitis C Virus Encoded Antigen (Recombinant c22-3, c200, and NS5), cat. no. 930740 (Ortho Diagnostic Systems, Inc. Raritan, NJ).

C. Reagents

Ortho HCV Version 3.0 ELISA Test System contains the following reagents; prepared by the manufacturer. Volumes listed are for 100-test / 1000-test kits.

- (1) Hepatitis C virus encoded antigen
5 microwell plates. Recombinant c22-3, c200, and NS5, coated microwell plate, 96 wells. c22-3, c200, and NS5 derived from yeast.

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- (2) Conjugate
1 bottle (125 mL). Antibody to human IgG – anti-human IgG heavy chain (murine monoclonal) conjugated to horseradish peroxidase with bovine protein stabilizers. Preservative: 0.02% thimerosal
- (3) Specimen diluent
1 bottle (190 mL). Phosphate-buffered saline with bovine protein stabilizers. Preservative: 0.1% 2-chloroacetamide.
- (4) o-Phenylenediamine · 2HCL
1 bottle. (OPD) tablets, 30 tablets.
- (5) Substrate buffer
1 vial (190 mL). Citrate-phosphate buffer with 0.02% hydrogen peroxide. Preservative: 0.01% thimerosal
- (6) Positive control
1 vial (1.0 mL). Inactivated human serum or plasma containing anti-HCV and non-reactive for hepatitis B surface antigen (HBsAg) and antibody to human immunodeficiency virus type 1 (HIV-1) and type 2 (HIV-2). Preservatives: 0.2% sodium azide and 0.9% EDTA
- (7) Negative control
1 bottle (1.5mL). Human serum or plasma nonreactive for HBsAg, antibody to HIV-1, antibody to HIV-2 and anti-HCV. Preservatives: 0.2% sodium azide and 0.9% EDTA
- (8) Plate sealers
21 plate sealers, disposable.

D. Reagent Preparation

- (1) 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. Prior to the second incubation transfer a sufficient volume of Substrate Buffer to a clean plastic container. Protect this reagent from light. Completely dissolve the appropriate number of OPD tablets in the Substrate Buffer prior to use.

Each microwell plate requires at least 20 mL of Substrate Solution. Guidelines for general use are given below.

Table 1
Guidelines for preparing Substrate Solution based on the number of wells to be tested.

Number of Wells	Number of Plates	Number of OPD Tablets	Substrate Buffer (mL)
24	0.25	1	6
48	0.5	2	12
72	0.75	3	18
96	1	4	24
192	2	7	42
288	3	10	60

The Substrate Solution is stable for 60 minutes after the addition of the OPD tablets when held in the dark at room temperature. The reagent should be colorless to very pale yellow when used. On the container record the time when the OPD tablets were added to the Substrate Buffer, and the time at which it will expire. If this reagent is noticeably yellow in color, discard and prepare fresh Substrate Solution as required.

- (2) Preparation of wash buffer (1X): Add 50 mL of 20X Wash Buffer Concentrate to 950 mL of distilled or deionized water. The 1 X Wash Buffer is stable for 30 days when stored at room temperature. The Wash Buffer is stable for up to 60 days when stored at 2 to 8°C. Record the preparation and expiration date the 1X Wash Buffer on the container. If the Wash Buffer is visibly contaminated it must be discarded.

Any lot number of 20X Wash Buffer Concentrate may be used to prepare the 1X reagent provided it is not used beyond its labeled expiration date.

E. Standards Preparation

There are no standards or calibrators in the conventional sense used with these kits. Calibration is based on the results of defined "positive" and "negative" controls.

- (1) Positive and negative control reagents are supplied with each test kit. The assay cutoff value is calculated from values obtained from these controls. The instrument automatically subtracts the blank value from each individual or sample value.
- (2) Calculation of negative control mean absorbance (NC_x)

Determine the mean of the negative controls.

Individual negative control values must be less than or equal to 0.120 and greater than or equal to -0.050. If one individual negative control value doesn't meet either of the criteria, exclude it and recalculate NC_x. All remaining individual control values must meet the above criteria, or the run should be repeated.

- (3) Calculation of positive control mean absorbance (PC_x)

Determine the mean of the positive controls.

Individual positive control values must meet the following criteria:

- (a) Individual control values must be greater than or equal to 0.800.
- (b) Individual control values should differ by no more than 0.600.

- (4) Assay run validity criteria

- (a) Individual positive controls must meet the criteria listed in Section 6.e.1. above.
- (b) Individual negative controls must meet the criteria listed in Section 6.e.2. above.
- (c) The substrate blank well must have an absorbance less than or equal to 0.050, and greater than or equal to -0.020.

- (5) An "in-house control" (IHC) is included with each testing series. The IHC reagent is purchased from Blackhawk Biosystems, Inc. (San Ramon, CA). Each time a new lot of reagent is purchased a mini-validation is performed. The control is run by multiple technicians on multiple days. The data is analyzed and an acceptable range is determined (+/- 3 standard deviations). This range is programmed into the HRL DMS so that each time run data is entered into the system the control

value is verified to be within the selected range. If it is within range the run is accepted, if not, then the run is not valid and not saved by the system.

F. Preparation of Quality Control Materials

- (1) Kit positive and negative controls are prepared and quality-controlled by the manufacturer.

6. 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

- (1) The instruments used to read assay results (Section 6.a.4.) are 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) Repeat positive specimens in duplicate. Repeatedly positive specimens are confirmed with RIBA 3.0 SIA.
- (3) Retest specimens having absorbance values below 0.005 using the same product and test method in order to verify the initial test result because poor technique may have caused the low values. If the specimen has an absorbance value less than the cutoff when retested, the specimen may be considered negative for antibodies by the criteria of the kit. Further testing is not required.
- (4) Specimens having absorbance values greater than or equal to cutoff are considered initially reactive by the criteria of the test kit. Before interpretation, however, retest the original sample in duplicate using the same product and test method. If either duplicate is reactive, the specimen may be interpreted to be repeatedly reactive for antibodies by the criteria of the test kit.
- (5) Retest initially reactive specimens must be repeated in duplicate using the same product and test method. If both of the duplicate repeat tests are negative, the test is considered negative by the criteria of the test kit.

7. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

A. Preliminaries

- (1) Reagents are used per kit of 480 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 reagents to warm to 20-25°C. Swirl gently before use. Adjust the incubator to 37°C ±2.
- (3) Assay the negative, positive, and in-house controls adjacent to the specimens using one bottle of diluted conjugate in the assay with each run of specimens. Use one preparation of working reagents per run.
- (4) Ensure that all reaction trays containing controls and samples 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.
- (7) Make certain that sufficient diluted conjugate is available for the test. If necessary, prepare additional conjugate reagent. Do not mix bottles of diluted conjugate.
- (8) Run separate positive and negative controls with each vial of diluted conjugate.
- (9) Follow the exact order of specimen and reagent addition as described in this test procedure.

B. Sample Preparation

- (1) Bring serum specimens to 20-25°C. While one box or rack of samples is being pipetted, the other racks should be refrigerated.
- (2) Serum and plasma samples may stratify when frozen or stored at 4-8°C for extended periods. Mix specimens gently before testing.
- (3) Identify the reaction tray wells for each specimen or 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.2.

- (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 HCV.
- (d) Press the Start button.
- (e) After the wash cycle is over remove the plate.
- (f) Set the AutoWash to Soak.

(2) Thermolyne incubator

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

(3) Operation of the Ortho AutoReader II

- (a) After the final reaction has been stopped, place the microwell plate on the carrier plate.
- (b) 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.
- (c) Select PLATE followed by GENERATE/EDIT A PLATE and press the ENTER key.
- (d) From the Assay column choose HCV 3.0 and enter a plate number and press ENTER
- (e) 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

- (f) Select RETURN TO MASTER MENU.
- (g) Go to the MASTER MENU and select READ A PLATE.
- (h) The Ortho AutoReader will automatically read the plate, and print the absorbance value for each well to a printer.

D. Operation of Assay Procedure

- (1) Pipet 200 μ L of Specimen Diluent to all wells of the microtiter platew except well 1A.
- (2) Pipet 10 μ L of controls or serum samples into the designated wells.
- (3) Apply cover seal. Incubate at 37 °C for 1 hour.
- (4) Place the microtiter plate on the AutoWash and wash all the wells five times with Wash Buffer (1X).
- (5) Add 200 μ L of Conjugate to all wells except 1A.
- (6) Apply cover seal. Incubate at 37 °C for 1 hour.
- (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 μ L of Substrate Solution to all the wells including 1A.
- (10) Apply cover seal. Incubate at room temperature for 30 min. in the dark.
- (11) Add 50 μ L 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.
- (14) Confirm repeatedly positive samples with the RIBA assay.

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 EIA, 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 Abbott 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 pipettor 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:

$$\text{Cutoff} = \text{NCx} + 0.600$$

- (2) Calculate the negative control mean absorbance (NCx) by determining the mean of the negative controls.

H. Special Procedure Notes

- (1) Do not splash liquid while tapping trays.
- (2) When washing beads, follow the directions provided with the washing apparatus.
- (3) When transferring the beads from the wells to the 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 the top of the tubes.
- (4) Avoid strong light during color development.
- (5) Dispense acid in the same sequence as the OPD substrate solution.
- (6) Do not allow acid or OPD substrate solution to contact metal.
- (7) Remove air bubbles prior to reading absorbance.
- (8) Visually inspect blank tubes and discard them if they have a yellow-orange color. If both tubes are contaminated, the entire run should be repeated.

8. REPORTABLE RANGE OF RESULTS

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

Specimens that repeatedly test positive are tested by RIBA 3.0 SIA for confirmation.

9. 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 in-house control anti-HCV positive serum is calibrated by serial dilution in a controlled process serum-based diluent. When tested by using the anti-HCV EIA, the final anti-HCV in-house control (IHC) reagent must consistently generate a signal-to-cutoff ratio of 1.5 ± 0.5 .

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-HCV 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 greater than or equal to the cutoff value are considered reactive for anti-HCV.

For the run to be valid, the difference between the mean absorbances 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 criterion:

Individual control values must be less than or equal to 0.120, and greater than or equal to -0.050.

An IHC is included with each testing series. The IHC reagent is purchased by the HRL and validated according to laboratory guidelines (SOP – W. Kuhnert, 1/04)

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.

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

- A. 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.
- B. If one negative or positive value fails to meet either of the acceptance criteria in the quality control procedures, it must be excluded and the NC_x or PC_x recalculated. All remaining individual control values must meet the criteria or the run should be repeated.
- C. For the run to be valid, the difference between the mean absorbances 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 repeated.
- D. 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 0.040. The user must determine assay validity due to substrate blank.

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.

11. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS

The sample is restricted to human serum or plasma. No interfering substances are identified. Closely monitor this procedure and the interpretation of results when testing serum or plasma specimens for the presence of antibody to HCV. Do not use heat-activated specimens. A negative test does not exclude the possibility of exposure to or infection with HCV. Negative results in this assay in individuals with prior exposure to HCV may be due to antibody levels below the limit of detection of the assay or lack of antibody reactivity to the HCV antigens used in this assay. Specimens may contain antibodies to either vector proteins or fusion proteins associated with the HCV recombinant antigens. Vector and/or fusion protein antibody-containing specimens may demonstrate reactivity that is unrelated to HCV infection. Additional, more specific, tests may be useful in defining the true HCV antibody reactivity.

12. REFERENCE RANGES (NORMAL VALUES)

All normal noninfected humans should have negative values for hepatitis C.

13. CRITICAL CALL RESULTS ("PANIC VALUES")

Not applicable.

14. SPECIMEN STORAGE AND HANDLING DURING TESTING

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

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

Other FDA-licensed tests for total anti-HCV 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.

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

Not applicable.

17. 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 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.

REFERENCES

1. Ortho Diagnostic Systems, Johnson & Johnson. Ortho HCV Version 3.0 Elisa Test System directional literature included with each assay kit: Ortho document #631-20-161-1, Ortho Diagnostic Systems, Raritan (NJ),1997.
2. Bradley DW. The agents of non-A, non-B hepatitis. *J Virol Meth* 1985;10:307-319.
3. Aach RD, Stevens CE, Hollinger B, et al. Hepatitis C virus infection in post-transfusion hepatitis: an analysis with first and second generation assays. *N Eng J Med* 1991;325:1325-9.
4. U.S. Department of Health and Human Services. Biosafety in microbiological and biomedical laboratories. HHS Publication (NIH) 88-8395. Washington:U.S. Government Printing Office, 1988.

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Confirmatory test - Chiron RIBA

1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

The Chiron RIBA HCV 3.0 Strip Immunoblot Assay (SIA) is an *in vitro* qualitative enzyme immunoassay for the detection of antibody to hepatitis C virus (anti-HCV) in human serum or plasma. Detection of anti-HCV by SIA methodology is based upon traditional Western and dot blotting techniques, in which specific immunogens (i.e. antigenic polyproteins) encoded by the HCV genome are immobilized onto a membrane support. Visualization of anti-HCV reactivity in specimens to the individual HCV-encoded proteins is accomplished using anti-human IgG enzyme-conjugates in conjunction with a colorimetric enzyme substrate. Qualitative determination of the human antibody directed against hepatitis C virus (anti-HCV) in human serum or plasma is measured using direct solid-phase enzyme immunoassay (1).

2. SAFETY PRECAUTIONS

Test kits for the strip immunoblot assay 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, test kit components should be treated as though they are capable of transmitting HCV. Consider all serum specimens for analysis potentially positive for infectious agents including HIV, hepatitis B virus and 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 #93-8395 be used by those handling test specimens and kit reagents (2).

Material safety data sheets for sodium azide, sulfuric acid, hydrochloric acid, o-phenylenediamine, and sodium hypochlorite are available through the National Center for Infectious Diseases computer network. Risk is minimal due to the small quantity of chemicals, the safety of packaging, and the limited handling by the operators using the test kits.

3. COMPUTERIZATION; DATA MANAGEMENT SYSTEM

- A. Raw data are transcribed manually from an instrument readout sheet into a computerized database or it can be uploaded in to the computerized database from a disk after the run information is exported from the instrument. 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" or "indeterminate" for RIBA. 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, 10/2002).
- 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 20 :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.

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- 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 $\leq -20^{\circ}\text{C}$. Samples held in long-term storage at $\leq -20^{\circ}\text{C}$ 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, CALBRATORS (STANDARDS), CONTROLS, AND ALL OTHER MATERIALS; EQUIPMENT AND INSTRUMENTATION

A. Instrumentation

- (1) RIBA Processor System (Chiron Corporation, Emeryville, CA).
- (2) RIBA Processor System Installation Kit Chiron Corporation, Emeryville, CA).
- (3) Gilson Pipetman micropipettors, 10 and 200 μL sizes (Rainin Instrument Co., Woburn, MA).

B. Materials

- (1) Deionized water (Continental Water Systems, Inc., San Antonio, TX).
- (2) Pipet tips, cat. nos. RT20 & RT200 (Rainin Instrument Co.).
- (3) Protective gloves, Tronex or Flexam, small/medium/large (Best Manufacturing, Menlo Park, GA).
- (4) 2 mL cryovials, cat. no. 5000-0020 (Nalge Company, Inc., Rochester, NY).
- (5) Cryovial boxes, cat. no. 5026-0909 (Nalge Company, Inc., Rochester, NY).
- (6) 1.5 mL microtubes (Marsh Biomedical Products, Rochester, NY).
- (7) 50 mL-polypropylene tubes (Corning Glass Works, Corning, NY).
- (8) Fixed or adjustable pipetting devices capable of delivering 20 ml and 1000 ml with at least $\pm 5\%$ accuracy (Gilson Pipetmen models P-20 and P-1000).
- (9) Forceps for handling strips (any vendor).
- (10) Chiron RIBA HCV 3.0 Strip Immunoblot Assay, Hepatitis C Virus Encoded Antigen/Peptide (Recombinant c33c and NS5 antigens; Synthetic 5-1-5, c100, and c22 peptides), cat. no. 930740 (Ortho Diagnostic Systems, Inc. Raritan, NJ).
- (11) RIBA Processor System (Ortho Diagnostic Systems, Raritan, NJ; product code 936595).

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(12) RIBA Processor System Installation Kit.

C. Reagents

Chiron RIBA HCV Version 3.0 Strip Immunoblot Assay kits contain the following reagents; prepared by the manufacturer. Volumes listed are for 30 tests.

- (1) Hepatitis C virus encoded antigen/peptide (Recombinant c33c and NS5; Synthetic 5-1-1p, c100p, and c22p) Coated strips
Each strip contains four individual bands coated with HCV-encoded antigens/peptides, a recombinant human SOD band, and two IgG control bands. 30 Consecutively numbered strips are provided. The automated version contains 3 sealed pouches, each with 10 strips in reaction vessels.
- (2) Conjugate
1 bottle (175 mL). Peroxidase-labeled goat anti-human IgG (heavy and light chains), with bovine protein stabilizers. Preservative: 0.01% thimerosal.
- (3) Specimen diluent
1 bottle (175 mL). Phosphate-buffered saline with bovine protein stabilizers and detergents. Preservative: 0.1% sodium azide and 0.05% gentamicin sulfate.
- (4) Substrate solution
1 bottle (17 ml). 4-chloro-1-naphthol-in methanol.
- (5) Substrate buffer
1 bottle (90 mL). Phosphate-buffered hydrogen peroxide.
- (6) Wash Buffer Concentrate (50X)
1 bottle (80). Phosphate-buffered detergent solution. Preservative: 0.01% thimerosal.
- (7) Positive control (Human)
1 vial (0.3 mL). Inactivated human serum or plasma containing antibodies to HCV (anti-HCV) and non-reactive for hepatitis B surface antigen (HBsAg) and antibody to human immunodeficiency virus type 1 (HIV-1) and type 2 (HIV-2). Preservatives: 0.1% sodium azide and 0.05% gentamicin sulfate.
- (8) Negative control (Human)
1 vial (0.3 mL). Human serum or plasma nonreactive for HBsAg, antibody to HIV-1, antibody to HIV-2 and anti-HCV. Preservatives: 0.1% sodium azide and 0.05% gentamicin sulfate.

D. Reagent Preparation

Bring the reagents to room temperature (15-30C°) and mix thoroughly by gently inverting the container several times. Avoid foaming.

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) The maximum run size is 30 strips (including a Positive and Negative control strip).

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- (2) The minimum run size is 3 strips (including a Positive and Negative control strip).
- (3) Each run must proceed to completion without interruption.
- (4) The positive and negative controls should be assayed with each series of patient/donor specimens. The positive and negative assays should be treated exactly as patient/donor specimens throughout the assay procedure.
- (5) An adequate amount of Working Wash Buffer and Working Substrate should be prepared before using the reagents.
- (6) If during the course of the assay it is determined that not enough Working Wash Buffer or Working Substrate has been prepared to complete the assay procedure, the assay run is considered invalid and must be repeated using a single preparation of the Working Solution.
- (7) Fading of the strips is prevented by keeping the developed strips out of strong light (direct sunlight) and away from heat (greater than 30 C).

B. Sample Preparation

- (1) Bring serum specimens to 20-25°C. While one box or rack of samples is being pipetted, the other racks should be refrigerated.
- (2) Serum and plasma samples may stratify when frozen or stored at 4-8°C for extended periods. Mix specimens gently before testing.
- (3) Identify the reaction tray wells for each specimen or control.

C. Instrument Setup

- (1) Turn on the instrument using the power switch located at the right rear of the instrument. The startup screen will be displayed and indicate that the instrument is proceeding with the system check. Within 5 minutes the main menu should be displayed indicating that the instrument has completed initialization.
- (2) From the Main Menu select Run Assay. The following steps will then need to be performed:
 - a) Prep: Select the assay to be run and the number of specimens and enter the kit lot and operator information.
 - b) Load Reagents: Load and verify the quantities of reagents loaded. The instrument will provide instructions on volume of reagents needed for the samples to be tested.
 - c) Load Reaction Vessels: Load reaction vessels (RVs) onto the carousel
 - d) Begin Run: Start the assay process.

D. Operation of Assay Procedure

The instrument performs the assay procedure.

E. Recording of Data

A NEGATIVE, INDETERMINATE, or POSITIVE interpretation is based on the reaction pattern present on the strip. For valid runs the following criteria should be used for interpretation:

Antigen Band Pattern	Interpretation
No HCV bands present having 1+ or greater reactivity Or hSOD band alone having 1+ or greater reactivity	NEGATIVE
Any <u>single</u> HCV band having 1+ or greater reactivity Or hSOD band having 1+ or greater reactivity in conjunction with one or more HCV bands having 1+ or grater reactivity	INDETERMINATE
At least <u>two</u> HCV bands having 1+ or greater reactivity	POSITIVE

A band intensity less than the IgG Control Level I (i.e., +/-) is below the cutoff for reactivity in the assay.

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A POSITIVE strip interpretation can only be made in the absence of reactivity to the hSOD band (i.e., - or +/-).

A POSITIVE test results indicates the presence of anti-HCV and past or present anti-HCV infection.

An INDETERMINATE test result indicates that anti-HCV may or may not be present and that a decision as to whether past or present HCV infection exists cannot be made. Since reactivity of 1+ or greater to any of the virus-encoded antigens on the strip is possible evidence of past or present HCV infection, all individuals who are INDETERMINATE should be retested again over a period of 6 to 12 months to ascertain whether increased reactivity has occurred. It is recommended that individuals who are INDETERMINATE be retested after six months using a freshly drawn specimen. A NEGATIVE test result by CHIRON RIBA 3.0 SIA which is REACTIVE by a licensed anti-HCV screening procedure does not exclude the possibility of infection with HCV. Levels of anti-HCV may be undetectable during the early stages of infection.

On rare occasions, a strip may have a dark background. If the Level I IgG and Level II IgG internal control bands are indistinguishable from the background (i.e., darker than the background, with the Level II IgG control darker than the Level I IgG control), the strip is interpretable and the intensity of the bands should be compared to the internal controls as described above. In anti-HCV negative specimens or specimens lacking antibodies to one or more antigens present on the strip, the antigen bands may appear lighter than the background of the strip. Such bands should be interpreted as nonreactive (i.e., - or +/-).

F. 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 the user 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

G. Calculations

The instrument performs any calculations that are needed.

H. Special Procedure Notes

None.

9. REPORTABLE RANGE OF RESULTS

Anti-HCV reactivity in a specimen is determined by comparing the intensity of each HCV band to the intensity of the human IgG (Level I and Level II) internal control bands on each strip. The identity of the antibodies is defined by the specified location of the HCV band as shown in Quality Control Procedures.

The intensity of the HCV bands is scored in relation to the intensity of the internal IgG controls as follows:

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Intensity of Band	Score
Absent	-
Less than intensity of the Level I IgG control band	+/-
Equal to intensity of the Level I IgG control band	1+
Greater than the intensity of the Level I IgG control band and less than the intensity of the Level II IgG control band	2+
Equal to intensity of the Level II IgG control band	3+
Greater than intensity of the Level II IgG control band	4+

10. QUALITY CONTROL (QC) PROCEDURES

- A. The assay controls supplied with the test kit must be included with each run, regardless of the number of specimens tested or strips used.
- B. The identity and location of the antigens coated on the strips are in the order below from the top of the strip (strip number) to the bottom.
- Strip number
 - IgG Control Level II
 - c-100(p);5-1-5(p)
 - c33c
 - c22(p)
 - NS5
 - hSOD
 - IgG Control Level I
- C. Two levels of human IgG (Level I, low control; and Level II, high control) are included on each strip as internal controls. The reactivity of the individual HCV bands is determined by comparing the intensity of each band to the Level I and Level II human IgG internal strip controls as described in Recording of Data.
- D. The following results are expected from the Positive and Negative Controls supplied with the test kit:
- (1) The internal IgG control Level I and control Level II on the Positive Control, Negative Control and each test specimen must be clearly distinguishable by eye, and the IgG control Level II must be visibly lighter than the IgG control Level I.
 - (2) The Positive Control strip must show a response of 2+ or greater for all HCV bands. Response to the hSOD band must be visibly lower than the Level I human IgG control (i.e., - or +/-).
 - (3) The Negative Control strip must show a response to each of the HCV and hSOD bands, which is visibly lower than the Level I, human IgG control (i.e., - or +/-).

If the assay kit controls do not meet the criteria above, then the run is invalidated and must be repeated.

Additionally, the IgG control Level I and control Level II bands must be clearly distinguishable by eye on all patient/donor specimen strips, and the IgG control Level I must be clearly lighter than the IgG control Level II. If these criteria are not met for an individual patient/donor specimen, the assay must be repeated for that specimen.

Note: If incomplete banding, or any such artifact, on a patient/donor specimen strip hinders interpretation, but the kit Positive and Negative control strips are interpretable, the patient/donor specimen strip is invalid and the assay must be repeated for that specimen.

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

Repeat run for individual sample as described above.

12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS

The sample is restricted to human serum or plasma. No interfering substances are identified. Closely monitor this procedure and the interpretation of results when testing serum or plasma specimens for the presence of antibody to HCV.

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Do not use heat-activated specimens. A negative test does not exclude the possibility of exposure to or infection with HCV. Negative results in this assay in individuals with prior exposure to HCV may be due to antibody levels below the limit of detection of the assay or lack of antibody reactivity to the HCV antigens used in this assay. Specimens may contain antibodies to either vector proteins or fusion proteins associated with the HCV recombinant antigens. Vector and/or fusion protein antibody-containing specimens may demonstrate reactivity that is unrelated to HCV infection. Additional, more specific, tests may be useful in defining the true HCV antibody reactivity.

13. REFERENCE RANGES (NORMAL VALUES)

All normal noninfected humans should have negative values for antibodies to hepatitis C.

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 up to 4 hours.

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

Other FDA-licensed tests for total anti-HCV may be substituted but must be accompanied by validation data to show substantial equivalence with this assay. 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 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.

REFERENCES

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