HEPATITIS C VIRUS (HCV)

Enzyme-Linked Immunosorbent Assay for the detection of antibody to Hepatitis C Virus (Anti-HCV) in human serum or plasma

ORTHO*
HCV
ELISA
TEST
SYSTEM

2nd GENERATION

*Trademark

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2nd Generation

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NAME AND INTENDED USE

ORTHO HCV ELISA Test System 2nd Generation is a qualitative, enzyme-linked, immunosorbent assay for the detection of antibody to hepatitis C virus (anti-HCV) in human serum or plasma.

SUMMARY AND EXPLANATION

The hepatitis C virus (HCV) is now believed to be the causative agent for most, if not all, blood-borne non-A, non-B hepatitis (NANBH).[1] Studies throughout the world indicate that HCV is transmitted through contaminated blood and blood products, through blood transfusions or through other close, personal contacts.[2] Currently in the United States, greater than 90% of transfusion-associated hepatitis infections are considered to be NANBH infections.[3] Worldwide, other forms of NANBH are recognized.

Recently, the RNA genome of the hepatitis C virus has been molecularly cloned, and an HCV-derived recombinant polypeptide, c100-3, has been used as the antigen in ORTHO* HCV ELISA Test System licensed for screening serum and plasma.[4] Recently, however, additional structural and nonstructural HCV antigens have been isolated and are designated as recombinants c22-3, a structural (core) protein, and c200, a nonstructural protein. These proteins are coated on microwells for use in ORTHO HCV ELISA Test System 2nd Generation.

The primary purpose of this assay is to screen blood donations for antibody to HCV. The hepatitis C virus encoded antigens used in the manufacture of ORTHO HCV ELISA Test System 2nd Generation are prepared by Chiron Corporation under a shared manufacturing arrangement.

PRINCIPLE OF THE PROCEDURE

The assay procedure is a three-stage test carried out in a microwell coated with a combination of recombinant hepatitis C virus (rHCV) antigens.

In the first stage, a test specimen is diluted directly in the test well and incubated for a specified length of time. If anti-HCV is present in the specimen, antigen-antibody complexes will be formed on the microwell surface. If anti-HCV is not present, complexes will not be formed and the unbound serum or plasma proteins will be removed in the subsequent washing step.

In the second stage, murine monoclonal antibody conjugate is added to the microwell. The conjugate binds specifically to the IgG anti-HCV portion of the antigen-antibody complexes. If antigen-antibody complexes are not present, the unbound conjugate will be removed by subsequent washing.

In the third stage, an enzyme detection system composed of OPD and hydrogen peroxide is added to the test well. If bound conjugate is present, the OPD will be oxidized, resulting in a colored end product. Sulfuric acid is then added to stop the reaction.

The color intensity is dependent upon the amount of bound conjugate and therefore is a function of the concentration of anti-HCV present in the specimen. The color intensity is measured with a microwell spectrophotometric reader (microwell reader).

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SPECIMEN COLLECTION AND PREPARATION

No special preparation of the patient/donor is required prior to specimen collection. Blood should be collected by approved medical techniques. Serum or plasma may be used and should be tested as soon as possible following collection. Do not use heat-treated specimens. Serum or plasma may be stored at 2 to 8°C for up to seven days. If longer storage is necessary, the specimens should be frozen (-20°C or lower) to limit possible contamination. Storage of specimens in self-defrosting freezers is not recommended. Avoid multiple freeze-thaw procedures. Mix specimen thoroughly after thawing and before testing. Clear, nonhemolyzed specimens are preferred; precipitates in specimens should be removed. All specimens should be handled as if capable of transmitting infectious agents. If specimens are to be shipped, they must be packed in compliance with federal regulations covering the transportation of etiologic agents.

PROCEDURE

Materials Provided

ORTHO HCV ELISA Test System 2nd Generation

480 Test Kit Components (Product code 03327/50575)

5 Hepatitis C Virus (HCV) Recombinant Antigen-Coated Microwell Plates (8 strips of 12 wells each in holder)

1 bottle Specimen Diluent (150 mL)—phosphate-buffered saline with protein stabilizers

1 bottle Conjugate: Antibody to Human IgG (Murine Monoclonal) (125 mL)—anti-human IgG (murine monoclonal) conjugated to horseradish peroxidase with protein stabilizers

1 vial OPD Tablets (30 tablets)—contains *o*-phenylenediamine·2HCl

1 bottle Substrate Buffer (155 mL)—citrate-phosphate buffer with 0.02% hydrogen peroxide

1 vial Positive Control (Human) (1.0 mL)
Source: Inactivated human serum or plasma containing anti-HCV and nonreactive for hepatitis B surface antigen (HBsAg) and antibody to human immunodeficiency virus type 1 (HIV-1)
Preservatives: 0.02% thimerosal and 0.1% sodium azide

1 vial Negative Control (Human) (1.5 mL)
Source: Human serum or plasma nonreactive for HBsAg, antibody to HIV-1 and anti-HCV
Preservatives: 0.02% thimerosal and 0.1% sodium azide

21 Plate sealers, disposable

CAUTION: HANDLE AS IF CAPABLE OF TRANSMITTING INFECTIOUS AGENTS.

Store at 2 to 8°C

For in vitro diagnostic use

MATERIALS REQUIRED BUT NOT PROVIDED

1. 50 µL to 300 µL adjustable 12-channel micropipette (Cat. No. 77-715-00; Flow Laboratories, McLean, VA) or equivalent reagent dispenser
2. 10 µL to 100 µL adjustable single-channel micropipette and disposable tips
3. Single-channel micropipette capable of delivering 200 µL and disposable tips or equivalent sample dilutor
4. 10 mL disposable serological pipettes
5. 5 µL to 300 µL disposable pipette tip bands (Cat. No. 77-987-H2; Flow Laboratories, McLean, VA) or equivalent
6. Multichannel micropipette reservoir (Cat. No. 77-824-00; Flow Laboratories, McLean, VA) or equivalent reagent container
7. Multichannel aspirator-washer device

8. Microwell spectrophotometric reader (microwell reader) capable of reading at 490 nm or 492 nm
9. 37°C incubator (dry or humidified)
10. Distilled or deionized water
11. 5.25% sodium hypochlorite (chlorine bleach)
12. 4N sulfuric acid (H_2SO_4) — available in United States from Ortho Diagnostic Systems (Product code 933740, Ortho Diagnostic Systems)
13. Black microwell strips (Ortho Diagnostic Systems)
14. 1 mL disposable graduated plastic pipettes
15. Vacuum source
16. Variable speed microwell plate shaker (Product code 933600, Ortho Diagnostic Systems) or equivalent, when manually delivering sample
17. 20X Wash Buffer Concentrate (Product code 03380/50580)

Precautions

1. CAUTION: Some components of this kit contain human blood components. No known test method can offer complete assurance that products derived from human blood will not transmit infectious agents. Therefore, all blood derivatives should be considered potentially infectious. It is recommended that these reagents and human specimens be handled using established good laboratory working practices.
2. Wear disposable gloves while handling kit reagents and specimens and thoroughly wash hands afterwards.
3. All specimens should be handled as potentially infectious agents.
4. Dispose of all specimens and materials used to perform the test as if they contained infectious agents.
5. Handle OPD tablets with plastic or Teflon®-coated forceps only. Metal forceps may react with tablets and interfere with the test results.
6. Avoid contact of OPD with eyes, skin or clothing, as OPD may cause irritation or an allergic skin reaction. If OPD should come into contact with the skin, wash thoroughly with water.
7. OPD tablets are light-sensitive and hygroscopic. Keep vial **tightly** closed when not in use and **bring to room temperature (15 to 30°C) before opening** the vial. The desiccant pouch must be retained in the vial at all times. Do not use tablets which are yellow or broken.
8. Distilled or deionized water must be used for Wash Buffer preparation. Water deionized with polystyrene resins may inactivate the horseradish peroxidase enzyme. Store the water in nonmetallic containers.
9. Do not mix reagents from kits with different lot numbers. Any lot number of 20X Wash Buffer Concentrate may be used provided it is not used beyond its labeled expiration date.
10. Cross-contamination between reagents will invalidate the test results. Permanently labeled, dedicated reservoirs for the appropriate reagents are recommended.
11. All reagents and components must be at room temperature prior to use.
12. All pipetting devices should be used with care and calibrated regularly, following the manufacturer's instructions.
13. When using a single-channel pipette for sample addition, use a new pipette tip for each specimen to be assayed. When using the multichannel micropipette, new tips are to be used for each specimen or reagent to be added.

14. The microwell strips are sealed in protective pouches with a humidity indicator desiccant. The desiccant, normally blue/purple in color, will turn pink if moisture is present in the pouch. If the desiccant is pink, the microwell strips should not be used.
15. Strict adherence to the specified wash procedure is crucial to ensure optimum assay performance (See Step 7 of Test Procedure).
16. Do not allow microwells to become dry once the assay has begun.
17. Do not touch the bottom exterior surface of the wells. Fingerprints or scratches may interfere with microwell reading.
18. Ensure that the microwell strips are level in the microwell strip holder. Before reading, wipe the bottom of the microwell strips carefully with a soft, absorbent tissue to remove any moisture.

Preparation of Reagents

1. **Preparation of Wash Buffer (1X):** Mix 50 mL of 20X Wash Buffer Concentrate with 950 mL distilled or deionized water. Wash Buffer is stable for 30 days when stored at room temperature. For longer storage (up to 60 days), store at 2 to 8°C. Discard Wash Buffer if visibly contaminated.

   NOTE: Any lot number of Wash Buffer Concentrate may be used to prepare this reagent provided it is not used beyond its labeled expiration date.

2. **Preparation of Substrate Solution:** Clean glass or plastic vessels must be used. Ten minutes prior to the end of the second incubation, transfer a sufficient amount of Substrate Buffer to a dark-colored or foil-covered vessel. Completely dissolve OPD tablets in Substrate Buffer.

Number of Wells	Number of OPD Tablets	Substrate Buffer (mL)
48	2	12
96	4	24
144	6	36
192	8	48

   The Substrate Solution is stable for 60 minutes at room temperature in the dark and should be clear to very pale yellow when used. If it is noticeably yellow in color, discard and prepare more Substrate Solution as required.

Test Procedure

1. Approximately 30 minutes prior to the beginning of the procedure, bring kit components to room temperature (15 to 30°C). Invert 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. One substrate blank, three negative controls and two positive controls will be included in each assay run in addition to specimens. If the entire strip is not needed, an appropriate number of wells can be broken off. Unused wells should be stored at 2 to 8°C in a tightly sealed foil pouch with desiccant and used within 5 days of opening the foil pouch.

   CAUTION: Handle microwell strips with care. Do not touch the bottom exterior surface of the wells.
   NOTE: After assay has begun, all steps must be completed without interruption.
3. Assemble the microwell strips in the microwell strip holder. Microwell strips must be level in the microwell strip holder. For incomplete 12-well rows, add black microwell strips.
4. Prepare a record identifying the placement of the controls and specimens in the microwells.

   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

5. Add controls and specimens to the microwells.
   a. When using automated instrumentation to deliver specimen to the microwell, follow the manufacturer's directions to achieve the appropriate volumes and dilutions required.
   b. When **manually** delivering the controls and specimens, the following steps should be performed.
      i) Add 200 µL of Specimen Diluent to all wells **except 1A.**
      NOTE: In the event reagent is added to well 1A, do not discard the run (See NOTE, Step 12).
      ii) Add 20 µL of the controls to the appropriate wells.
      iii) Add 20 µL of each serum or plasma specimen to be tested to the appropriate wells.
      iv) Place the microwell strip holder on a microwell plate shaker to mix for 5 to 10 seconds. The shaker should be used at a slow to moderate speed, taking care to avoid splashing of the contents of the test wells.
6. Cover the microwell strip holder with a plate sealer and incubate at 37°C (± 1°C) for 60 minutes (± 5 minutes).
7. With an aspirator-washer device, aspirate and wash all wells five times with Wash Buffer (1X).

   CAUTION: Strict adherence to the specified wash procedure is crucial to ensure optimum assay performance. Follow the steps specified in order to ensure thorough washing.
   a. Aspirate the sample solutions from microwells then fill completely with Wash Buffer (1X). Do not allow the wells to overflow. Allow approximately 20 seconds between the addition of Wash Buffer and subsequent aspiration.
   b. Complete the aspirate/fill sequence four additional times.
   c. Completely aspirate wells. Invert the plate and firmly tap on a clean paper towel to remove excess Wash Buffer.

   NOTE: Decontaminate liquid waste using sodium hypochlorite prior to disposal.
8. Using a multichannel micropipette, add 200 µL of Conjugate to all wells **except 1A** (See NOTE, Step 12).
9. Cover the microwell strip holder with a new plate sealer and incubate at 37°C (± 1°C) for 60 minutes (± 5 minutes).
10. Prepare Substrate Solution 10 minutes prior to the end of the second incubation. Refer to Preparation of Reagents.
11. After the second incubation, wash the wells as described in Step 7.
12. Using a multichannel micropipette, add 200 µL of Substrate Solution to all wells, **including 1A.**

    NOTE: If reagent was inadvertently added to well 1A in Step 5 and/or Step 8, add 200 µL of Substrate Solution to an empty well and use that well as the blank in Step 15. This new blanking well must be detached and placed in the 1A position when attempting to read the plate.
13. Incubate at room temperature in the dark for 30 minutes (± 1 minute).
14. Using a multichannel micropipette, forcibly add 50 µL of 4N sulfuric acid (H_2SO_4) to all wells, including 1A. Ensure thorough mixing of reagents.

15. Wipe the bottom of the microwell strips carefully with a soft, absorbent tissue to remove any moisture before reading. Read the microwell strip plate at a wavelength of 490 nm or 492 nm. For dual wavelength readers set the reference wavelength at 620 nm or 630 nm. Blank the reader on well 1A according to manufacturer's instructions.

NOTE: Microwell strip plates must be read within 60 minutes following the addition of 4N sulfuric acid. Plates must be stored in the dark until read.

IMPORTANT: AT THE END OF EACH DAY, FLUSH THE WASHER WITH 500 mL OF DISTILLED WATER.

Calculation of Results

1. Calculation of Negative Control Mean ($NC\bar{x}$)
   a. Individual negative control absorbance values must be less than or equal to 0.150 and greater than or equal to −0.005. For negative control values between 0.000 and −0.005, round to 0.000. If one of the three control values is outside either of these limits, recalculate the negative control mean based upon the two acceptable control values. The assay is invalid and the test must be repeated if two of the three control values are outside either of the limits.
   b. Determine the mean of the negative control values.

   Example:

Negative Control	Absorbance
1	0.040
2	0.050
3	0.060

Total Absorbance = 0.150

$$NC\bar{x} = \frac{\text{Total Absorbance}}{3} = 0.050$$

2. Calculation of Positive Control Mean ($PC\bar{x}$)
   a. Individual positive control absorbance values must be greater than or equal to 0.600.
   b. Determine the mean of the two positive control values as was done for the negative control values.
   c. Individual positive control values must be within the range of 0.5 to 1.5 times the mean of the positive control. If one value falls outside this range, the assay is invalid and must be repeated.
3. Calculation of the Cutoff Value

   Cutoff Value = $NC\bar{x} + 0.400$

   Example:

Negative Control	Absorbance
1	0.040
2	0.050
3	0.060

Total Absorbance = 0.150

$$NC\bar{x} = \frac{\text{Total Absorbance}}{3} = 0.050$$

Cutoff Value = 0.050 + 0.400 = 0.450

INTERPRETATION OF RESULTS

1. Specimens with absorbance values less than the Cutoff Value are considered negative (nonreactive). Further testing is not required.
2. Specimens with absorbance values greater than or equal to the Cutoff Value are considered initially reactive and should be retested in duplicate before final interpretation.
3. Upon retesting an initially reactive specimen, the specimen is considered reactive for antibody to HCV if either or both duplicate determination(s) is (are) reactive, i.e., greater than or equal to the Cutoff Value.
4. After retesting an initially reactive specimen, the specimen is considered nonreactive for antibody to HCV if both duplicate determinations are negative, i.e., less than the Cutoff Value.

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ORTHO HCV ELISA Test System
2nd Generation
Quick Reference Procedure

1. Bring all reagents to room temperature (15 to 30°C).
2. Prepare record for identifying placement of controls and specimens in microwells.
3. Assemble microwells in strip holder.
4. Pipette 200 µL of Specimen Diluent to all wells **except 1A.**
5. Pipette 20 µL of controls and serum or plasma specimen into microwells.
6. When specimens are added manually, place microwell strip holder on a microwell plate shaker to mix for 5 to 10 seconds after specimen addition.
7. Incubate for 60 minutes at 37°C.
8. Wash five times with Wash Buffer (1X).
9. Pipette 200 µL of Conjugate to all wells **except 1A.**
10. Incubate for 60 minutes at 37°C.
11. Prepare fresh Substrate Solution 10 minutes prior to the end of the second incubation.
12. Wash five times with Wash Buffer.
13. Pipette 200 µL of Substrate Solution to all wells **including 1A.**
14. Incubate for 30 minutes at room temperature in the dark.
15. Forcibly add 50 µL of 4N H_2SO_4 to **all wells.**
16. Wipe outside bottom of microwells with tissue.
17. Measure the absorbance of each well at 490 nm or 492 nm.

IMPORTANT: AT THE END OF EACH DAY, FLUSH THE WASHER WITH 500 mL of DISTILLED WATER.

Preparation of Substrate

Number of Wells	Number of OPD Tablets	Substrate Buffer (mL)
48	2	12
96	4	24
144	6	36
192	8	48

Manufactured by
Ortho Diagnostic Systems N.V.
A Johnson & Johnson COMPANY · 2340 BEERSE, BELGIUM

Recombinant Antigens
Provided by
CHIRON
CORPORATION EMERYVILLE, CA 94608

01082
Issued March 1991