

Laboratory Procedure Manual

Analyte: **Hepatitis C RNA Qualitative Assay for
Hepatitis C Virus**

Matrix: **Serum**

Method: **AMPLICOR Hepatitis C Virus (HCV) Test, v2.0**

Method No.:

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As performed by: Assay Development and Hepatitis Reference Laboratory (ADHRL)
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Important Information for Users

The National Center for HIV/AIDS, Hepatitis, STD and TB Prevention (NCHHSTP) 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
HEPC_F	LBXHCR	Hepatitis C RNA (HCV-RNA)

AMPLICOR[®] Hepatitis C Virus (HCV) Test, version 2.0

LABORATORY PROCEDURE MANUAL

Hepatitis C Virus RNA in Human Serum or EDTA Plasma

RECORD OF CHANGES: All changes should be made in the space provided or on the corresponding facing page. Each change or notation should be referenced to the appropriate paragraph number and signed by the director.

REVIEW POLICY: This procedure manual is reviewed by the director annually and at other times as required by major changes in procedure or other circumstances affecting laboratory performance of the tests herein described.

NOTE: This Laboratory Procedure Manual contains the most current information available at the time of printing. Refer to the date at the top of the page for the package insert revision used. Therefore, the Laboratory Procedure Manual should not be considered a substitute for the most current revision of the AMPLICOR Hepatitis C Virus (HCV) Test, version 2.0, package insert. Always refer to the package insert for the most up-to-date information.

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1. Principle of Test

General Testing Principle

- 1.1 The AMPLICOR HCV Test, v2.0 is based on five major processes: specimen preparation, reverse transcription to generate cDNA from target HCV RNA and HCV Internal Control (HCV IC) RNA, PCR amplification of target cDNAs using HCV-specific primers, hybridization of the amplified cDNAs to target-specific oligonucleotide probes, and colorimetric detection of the probe-bound amplified cDNAs.

The AMPLICOR HCV Test, v2.0 permits the simultaneous reverse transcription and PCR amplification of HCV and HCV IC target RNAs. The Master Mix reagent contains a primer pair that is specific for both HCV and HCV IC RNAs. Detection of amplified DNA is performed using target-specific oligonucleotide probes that permit independent identification of HCV amplicon and HCV IC amplicon.

Appropriate selection of primers and probe was critical for AMPLICOR HCV Test, v2.0 detection of all recognized genotypes. Accordingly, selection of the target RNA sequence was based on identifying a region of the HCV genome that was maximally conserved among genotypes. HCV RNA sequences are most conserved in the 5' untranslated region (UTR). The AMPLICOR HCV Test, v2.0 uses primers KY78 and KY80 to amplify a 5' UTR sequence of 244 nucleotides. HCV RNA sequence corresponding to these primers and the capture probe are located in the most conserved 5' UTR domains.

Specific Test Processes

- 1.2 Specimen Preparation

HCV RNA is isolated directly from serum or EDTA plasma by lysing virus particles with a chaotropic agent. HCV IC RNA, introduced into each specimen with Lysis Reagent, serves as an extraction and amplification control for each processed specimen. HCV and HCV IC RNAs are precipitated by using alcohol and then resuspended in Specimen Diluent.

- 1.3 Reverse Transcription and PCR Amplification

Reverse transcription and amplification reactions are performed with the thermostable recombinant enzyme *Thermus thermophilus* DNA Polymerase (*rTth* pol). In the presence of manganese (Mn^{2+}) and the appropriate buffer, *rTth* pol has reverse transcriptase and DNA polymerase activities. This allows both reverse transcription and PCR amplification to occur in the same reaction mixture.

Processed specimens are added to the amplification mixture in amplification tubes (A-tubes) in which reverse transcription and PCR amplification occur. The downstream or antisense primer (KY78) is biotinylated at the 5' end; the upstream or sense primer (KY80) is not biotinylated. The reaction mixture is heated in the thermal cycler analyzer to allow specific annealing of the downstream primer to target HCV and HCV IC RNAs. In the presence of Mn^{2+} and excess deoxynucleoside triphosphates (dNTPs), including deoxyadenosine, deoxyguanosine, deoxycytidine, and deoxyuridine (in place of thymidine) triphosphates, *rTth* pol extends the annealed primer to form cDNA.

Target Amplification

Following reverse transcription of target HCV and HCV IC RNAs, the reaction mixture is heated to denature RNA:cDNA hybrids and expose sequences that anneal with the primers. As the mixture cools, the upstream primer (KY80) anneals specifically to the cDNA strand representing each target RNA, *rTth* pol extends the primer and a second DNA strand is synthesized. This completes the first cycle of PCR, yielding a double-stranded DNA copy of each RNA target region (HCV and HCV IC).

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The reaction mixture is heated again to separate the double-stranded DNA and expose the primer-annealing sequences. As the mixture cools, primers KY78 and KY80 anneal to target DNA. The *rTth* pol enzyme, in the presence of Mn^{2+} and excess dNTPs, extends the annealed primers along the target templates to produce a 244-base pair double-stranded DNA "amplicon." The thermal cycler automatically repeats this process for 37 cycles, with each cycle intended to double the amount of amplicon DNA. The required number of cycles is preprogrammed into the thermal cycler. Amplification occurs only in the region of the HCV genome between the primers; the entire genome is not amplified.

HCV Internal Control (HCV IC) Amplification

In enzyme-based amplification processes such as PCR, inhibitors that may be present in the clinical specimen can reduce amplification efficiency. The HCV IC has been added to the AMPLICOR HCV Test, v2.0 to permit identification of processed specimens containing substances that may interfere with PCR amplification or specimens in which HCV RNA may have been lost during specimen processing. The HCV IC is an RNA transcript with primer-annealing regions identical to those in the HCV genome, a randomized internal sequence of similar length and base composition as the HCV target sequence, and a unique probe-binding region that differentiates HCV IC amplicon from HCV amplicon. These features were selected to ensure equivalent amplification of HCV IC and HCV target RNAs. The HCV IC is introduced into each specimen with the Lysis Reagent and serves as an extraction and amplification control for each processed specimen.

Selective Amplification

Selective amplification of target nucleic acid from the clinical specimen is achieved in the AMPLICOR HCV Test, v2.0 by the use of AmpErase® (uracil-N-glycosylase) and deoxyuridine triphosphate (dUTP). AmpErase recognizes and catalyzes the destruction of DNA strands containing deoxyuridine, but not DNA containing deoxythymidine. Deoxyuridine is not present in naturally occurring DNA. It is always present in amplicon due to the use of deoxyuridine triphosphate in place of thymidine triphosphate as one of the dNTPs in the Master Mix reagent; therefore, only amplicon contains deoxyuridine. Deoxyuridine renders contaminating amplicon susceptible to destruction by AmpErase prior to the amplification of target DNA. AmpErase, which is included in the Master Mix reagent, catalyzes the cleavage of deoxyuridine-containing DNA at the deoxyuridine residues by opening the deoxyribose chain at the C1-position. When heated in the first thermal cycling step at the alkaline pH of Master Mix, the amplicon DNA chain breaks at the position of the deoxyuridine, thereby rendering the DNA non-amplifiable. AmpErase is inactive at temperatures above 55°C; therefore, it does not destroy target amplicon during the thermal cycling steps. Following amplification, any residual AmpErase is denatured by the addition of the Denaturation Solution, thereby preventing the degradation of any target amplicon.

1.4 Hybridization Reaction

Following PCR amplification, and after the addition of Denaturation Solution to the reaction tubes, the HCV and HCV IC amplicon are chemically denatured to form single-stranded DNA. Aliquots of denatured amplicon are then transferred to separate wells of microwell plates (MWP) coated with either an HCV-specific (KY150) or HCV IC-specific (SK535) oligonucleotide probe. The biotin-labeled HCV and HCV IC amplicon are hybridized to the target-specific oligonucleotide probes bound to the wells of the MWP. This hybridization of amplicon to the target-specific probe increases the overall specificity of the test.

1.5 Detection Reaction

Following the hybridization reaction, the MWP is washed to remove unbound material and then Avidin-Horseradish Peroxidase Conjugate is added to each well of the MWP. The conjugate binds to the hybridized biotin-labeled HCV and HCV IC amplicons. The MWP is washed again to remove unbound conjugate and then a substrate solution containing hydrogen peroxide and 3,3',5,5'-tetramethylbenzidine (TMB) is added to each well. In the presence of hydrogen peroxide, bound horseradish peroxidase catalyzes the oxidation of TMB to form a colored complex. The reaction is stopped by addition of a weak acid and the absorbance of the wells is measured at 450nm (A_{450}) using a microwell plate reader.

2. Clinical Significance

- 2.1 *HCV causes the most common chronic parenterally transmitted infection in the United States. It is estimated that approximately 1.8% of Americans and 0.6% of Canadians have been infected with HCV. HCV-associated end-stage liver disease is the most frequent indication for liver transplantation among adults, and the Centers for Disease Controls and Prevention (CDC) estimate that 8,000-10,000 deaths per year are due to HCV-related chronic liver disease. CDC produced recommendations in 1998 outlining which persons should routinely be tested for HCV infection. These include, but are not limited to, persons with persistently abnormal alanine transaminase (ALT) levels, persons who ever injected illegal drugs, certain prior recipients of blood or blood components or an organ transplant, and persons who were ever on chronic hemodialysis. Persons with recognized exposure risk (such as healthcare or emergency workers in contact with blood, or babies born to HCV-infected women) should also be tested.*
- 2.2 Available diagnostic tests either detect direct antibodies to HCV (anti-HCV) or HCV RNA. Anti-HCV indicates prior exposure to HCV but does not distinguish between cleared and active infection (i.e., the virus is replicating). In a person with anti-HCV, detectable HVC RNA indicates active infection. The results of HCV RNA testing can identify patients with active infection and, together with other biochemical and clinical information, may be used to provide counseling and assess whether treatment is appropriate. Following diagnosis, HCV-infected persons can be counseled about protecting the liver from further harm and reducing risk for transmission to others. They can also be advised regarding assessment of liver function and disease severity and available treatment options.

3. Specimen Requirements

Specimen Collection

Note: Handle all specimens as if they are capable of transmitting infectious agents.

- 3.1 No special patient preparation before collection is necessary.
- 3.2 *Blood should be collected in SST[®] Serum Separation Tubes, in sterile collection tubes with no additives (red tops), or in sterile tubes using EDTA. **Specimens collected using heparin as the anticoagulant are unsuitable for this test.***
- 3.3 *Collect one SST, one red top or one EDTA tube using standard venipuncture techniques. Follow the manufacturer's instructions for use of the collection tubes.*
- 3.4 Specimen collection supplies are located:

Refer to sample collection SOP.

Specimen Identification

- 3.5 Label the specimen container with the patient's name or identification number and the date.
- 3.6 Record the patient's name or identification number, date, and test(s) requested in the Laboratory Information System (LIS) or in the Test Log Book.

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Specimen Transport

- 3.7 *Transportation of whole blood, serum or EDTA plasma must comply with country, federal, state and local regulations for the transport of etiologic agents. Whole blood must be transported at 2 -- 25°C and processed within six hours of collection. Serum or EDTA plasma may be transported at 2 -- 8°C for 72 hours or frozen at -70°C or colder indefinitely.*

Specimen Handling and Storage

- 3.8 *Separate serum or EDTA plasma from whole blood within six hours of collection by centrifugation at 1500 x g for 20 minutes at room temperature.*
- 3.9 *Avoid prolonged contact of serum or EDTA plasma with the red blood cells. Immediately transfer serum or EDTA plasma to a properly identified, sterile, screw-cap, polypropylene tube after centrifugation.*
- 3.10 *Serum or EDTA plasma specimens may be stored at 2 -- 8°C for up to 72 hours or frozen at -70°C or colder indefinitely. Serum or EDTA plasma specimens may be frozen and thawed up to three times without a loss of HCV RNA.*
- 3.11 *Treat all specimens as potentially infectious. Wear gloves and protective clothing and promptly clean up all spills. Dispose of specimens and specimen containing reagents in marked biohazard containers.*

4. Reagents

Reagent Composition

- 4.1 The AMPLICOR HCV Test, version 2.0 kits include the following reagents:

Specimen Preparation Kit		
HCV Lysis Reagent, version 2.0	HCV LYS, v2.0	Tris-HCl buffer containing 68% guanidine thiocyanate, 3% dithiothreitol, and <1% glycogen
HCV Internal Control, version 2.0	HCV IC, v2.0	<0.001% Non-infectious <i>in vitro</i> transcribed RNA (microbial) containing HCV primer binding sequences and a unique probe binding region <0.005% poly rA RNA (synthetic), EDTA and 0.05% sodium azide.
HCV Specimen Diluent, version 2.0	HCV DIL, v2.0	Tris-HCl buffer containing EDTA, <0.005% poly rA RNA, and 0.05% sodium azide.

Amplification Kit		
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HCV Master Mix, version 2.0	HCV MMX, v2.0	Bicine buffer containing 16% DMSO, glycerol, potassium acetate <0.001% dATP, dCTP, dGTP and dUTP, <0.005% KY78 & KY80 primers, <0.01% <i>rTth</i> Pol, <0.01% AmpErase® (microbial), and 0.05% sodium azide
HCV Manganese Solution, version 2.0	HCV Mn ²⁺ , v2.0	<2% manganese, acetic acid, amaranth dye and 0.05% sodium azide

Control Kit		
Negative Human Plasma	NHP	Human plasma, non-reactive by US FDA licensed tests for antibody to HIV-1 and HIV-2, antibody to HCV, HIV p24 antigen and HbsAg, and 0.1% ProClin® 300.
HCV Negative Control, version 2.0	HCV (-) C, v2.0	<0.005% Poly rA RNA (synthetic), EDTA and 0.05% sodium azide
HCV Positive Control, version 2.0	HCV (+) C, v2.0	<0.001% Non-infectious <i>in vitro</i> transcribed RNA (microbial) containing HCV sequences, <0.005% poly rA RNA (synthetic), EDTA, and 0.05% sodium azide.

HCV Detection Kit		
HCV Microwell Plate, version 2.0	HCV MWP, v2.0	MWP coated with HCV-specific DNA probe (KY150) Twelve, 8-well strips in one resealable pouch with desiccant.
Denaturation Solution	DN	1.6% Sodium hydroxide, EDTA, Thymol blue

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HCV Hybridization Buffer	HCV HYB	Sodium phosphate solution containing <0.2% solubilizer and <25% sodium thiocyanate.
Avidin-Horseradish Peroxidase Conjugate	AV-HRP	Tris-HCl buffer containing <0.001% avidin-horseradish peroxidase conjugate, bovine gamma globulin (mammalian), Emulsit 25(Dai-ichi Kogyo Seiyaku Co., Ltd.), 0.1% phenol, 1% ProClin® 150
Substrate A	SUBA	Citrate solution containing 0.01% hydrogen peroxide and 0.1% ProClin 150
Substrate B	SUBB	0.1% 3,3',5,5'-tetramethylbenzidine (TMB) and 40% dimethylformamide (DMF)
Stop Reagent	STOP	4.9% Sulfuric acid
10X-Wash Concentrate	10X WB	<2% Phosphate buffer, <9% sodium chloride, EDTA, <2% detergent, and 0.5% ProClin 300

AMPLICOR Internal Control Detection Kit		
Internal Control Microwell Plate	IC MWP	MWP coated with IC-specific DNA probe (SK535). Twelve 8-well strips in one resealable pouch with desiccant.
Avidin-Horseradish peroxidase conjugate	AV-HRP	Tris-HCl buffer containing <0.001% avidin-horseradish peroxidase conjugate, bovine gamma globulin (mammalian), Emulsit 25(Dai-ichi Kogyo Seiyaku Co., Ltd.), 0.1% phenol, 1% ProClin® 150
Substrate A	SUBA	Citrate solution containing 0.01% hydrogen peroxide and 0.1% ProClin 150
Substrate B	SUBB	0.1% 3,3',5,5'-tetramethylbenzidine (TMB) and 40%

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		dimethylformamide (DMF)
Stop Reagent	STOP	4.9% Sulfuric acid
10X–Wash Concentrate	10X WB	<2% Phosphate buffer, <9% sodium chloride, EDTA, <2% detergent, and 0.5% ProClin 300

Special Supplies

4.2 The following supplies are needed but not supplied in the AMPLICOR HCV Test, version 2.0 kit:

Pre-Amplification—Reagent Preparation Area	Disposable, powderless gloves, lab coat
	MicroAmp [®] Reaction Tubes, Tray/Retainers and Base.
	Plastic resealable bag
	Eppendorf Repeater [®] pipet with 1.25 mL Combitip [®] Reservoir (sterile, individually wrapped)
	Pipettors (capacity 50 & 100 μ L) with aerosol barrier or positive displacement RNase-free tips <i>Pipettes should be accurate within 3% of stated volume. Aerosol barrier or positive displacement RNase-free tips must be used to prevent specimen and amplicon cross contamination.</i>

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Pre-Amplification—Specimen Preparation Area	Disposable, powderless gloves, lab coat
	Microcentrifuge (max. RCF 16,000 x g, min. RCF 12,500 x g); Eppendorf 5415C, HERMLE Z230M, or equivalent
	1.5 mL polypropylene screw-cap tubes, sterile, non-siliconized, conical (Sarstedt 72.692.005 or equivalent)
	Tube racks (Sarstedt 93.1428 or equivalent)
	60°C ± 2°C dry heat block
	95% ethanol, reagent grade for microbiology or Histology use (freshly diluted to 70% using distilled or deionized water)
	Isopropanol, reagent grade
	Sterile, transfer pipets, RNase-free

	Vortex mixer
	Sterile, disposable, polystyrene serological pipets (5, 10 and 25 mL)
	Pipettors (capacity 20, 50, 100, 200, 400, 600 and 1000 µL) with aerosol barrier or positive displacement RNase-free tips <i>Pipettes should be accurate within 3% of stated volume. Aerosol barrier or positive displacement RNase-free tips must be used to prevent specimen and amplicon cross contamination.</i>

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Post Amplification Area— Amplification/Detection	Disposable, powderless gloves, lab coat
	Multichannel pipettor (capacity 25 and 100 μ L) or electronic pipettor (Impact [®] or AMPLICOR [®])
	Aerosol barrier or positive displacement RNase-free tips (25 and 100 μ L) and barrier-free tips (100 μ L)
	Applied Biosystems GeneAmp PCR System 9600 or GeneAmp PCR System 2400 thermal cycler
	MicroAmp base and cap installing for use with Applied Biosystems GeneAmp PCR System 9600 or GeneAmp PCR System 2400
	Microwell plate washer <i>Capable of washing 12 x 8 microwell format with 250-300 μL of Wash Solution per well at 30 second time intervals.</i>
	Microwell plate reader <i>Specifications: Bandwidth = 10 nm \pm 3 nm; Absorbance range = 0 to \geq 3.00 A_{450}; Repeatability \leq 1%; Accuracy \leq 3% from 0 to 2.00 A_{450}; Drift \leq 0.01 A_{450} per hour.</i>
	Disposable reagent reservoirs
	Microwell plate lid
	96-well strip ejector, Costar [®] #2578
	Incubator 37°C \pm 2°C
	5 mL serological pipets

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	Graduated cylinder
	Distilled or deionized water

Reagent Labeling and Preparation

- 4.3 All reagents are labeled by the manufacturer. The labeling includes contents, lot number, expiration date, and storage instructions.
- 4.4 All reagents are liquid, ready-to-use. Working reagents are prepared at specific intervals during the test process. These are:

Working Master Mix: Prepare by adding 100 μ L HCV Mn²⁺ to one vial HCV MMX, v2.0, store at 2–8°C, use within 4 hours of preparation. Quantity sufficient for 12 reactions.

Working Lysis Reagent: Prepare by adding 100 μ L HCV IC, v2.0 to one bottle HCV LYS, v2.0, store at room temperature, use within 8 hours of preparation.

Working CX4, v2.0 Reagent: Prepare by adding 2.5 mL CX PS1, v2.0 to the CX4, v2.0 cassette; stable for 30 days at 2–8°C; may be used for a maximum of 6 instrument cycles (12 hours per cycle) and must be stored at 2–8°C between cycles.

Working IC4 Reagent: Prepare by adding 2.5 mL IC PS1 to the IC4 cassette; stable for 21 days at 2–8°C; may be used for a maximum of 6 instrument cycles (12 hours per cycle) and must be stored at 2–8°C between cycles.

Working Substrate: Prepare by adding 5 mL SB to one SB3 cassette; stable on the analyzer for 16 hours; must be prepared daily; protect from direct light and exposure to metals or oxidizing agents.

Working Wash Solution: Prepare by adding 1 volume 10X-Wash Concentrate to 9 volumes distilled water; store at 2–25°C in the COBAS AMPLICOR Wash Buffer Reservoir (Do not top off the reservoir.); use within two weeks of preparation.

Reagent Storage and Use

- 4.5 *Store the following reagents at 2–8°C: HCV LYS, v2.0; HCV IC, v2.0; HCV MMX, v2.0; HCV Mn²⁺, v2.0; NHP; HCV (-) C, v2.0; HCV (+) C, v2.0; HCV DIL; HCV MWP, v2.0; IC MWP; AV-HRP; SUBA and SUBB.*

*Store the following reagents at 2 -- 25°C: DN, HCV HYB, STOP and WB.
Do not freeze reagents.*

- 4.6 Do not use reagents beyond the expiration dates shown on the packages. All reagents are stable to the expiration date.

The AV-HRP, SUBA and SUBB are stable for 3 months or the expiration date, whichever comes first, at 2–8°C once opened.

The HCV MWP and IC MWP are stable for 3 months or until the expiration date, whichever comes first, at 2–8°C once opened. Store MWPs in resealable pouches containing desiccant.

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The DN and STOP are stable for at least 5 months at 2–25°C once opened.

The HCV HYB is stable for 30 days at 2–8°C once opened.

10XWB is stable for at least 5 months at 2–25°C once opened.

The stability of the Working Reagents is noted with their instructions for preparation—See Section 4.4.

- 4.7 Do not mix reagents from different lot numbers. Do not pool reagents. Dispose of unused reagents and waste in accordance with all local, country, state, and federal regulations.
- 4.8 Handle all reagents with caution and avoid contact with skin, eyes, or mouth. Refer to the package insert for any known toxicity.
- 4.8.1 These reagents contain **sodium azide**: HCV MMX, v2.0; HCV Mn²⁺, v2.0; HCV DIL, v2.0; HCV IC, v2.0; HCV (-) C, v2.0; HCV (+), v2.0. Avoid swallowing and contact with skin and mucous membranes. Sodium azide may react with lead and copper plumbing to form highly explosive metal azides. On disposal, flush with a large amount of water to prevent azide build-up.
- 4.8.2 These reagents contain **dimethylformamide**: Substrate B and Working Substrate. Dimethylformamide is an irritant and has been reported to be teratogenic in high oral doses. Wear gloves when handling these reagents. Avoid skin contact, inhalation of fumes and ingestion. May cause harm to the unborn child.
- 4.8.3 These reagents carry a **burn warning**: Denaturation and Stop Solutions. Avoid contact of these materials with the skin, eyes, and mucous membranes. If contact does occur, immediately wash with large amounts of water. If spills of these reagents occur, dilute with water before wiping dry.
- 4.8.4 These reagents are marked as **harmful or irritants**: HCV LYS, v2.0 and HYC HYB, v2.0.
- 4.9 Reagents required to perform this assay are located in the flammable storage refrigerator (model 3551-10) along with other assay reagents in room 1-1383.

5. Test Procedure

Run Size and Workflow

- 5.1 *Each kit contains sufficient reagents for eight 12-specimen runs. The Specimen Preparation Reagents and Amplification Reagents are packaged in 12-test, single-use bottles. Therefore, it is recommended that processing should be in batches of multiples of 12 for the most efficient use of reagents, specimens, and controls.*

Examine all reagents for sufficient volume before beginning the test process. (See Section 4.7.)

- 5.2 *The AMPLICOR HCV Test, v2.0 may be completed in one day or over two days. Testing can be completed over 2 days, by starting Specimen and Control Preparation on day 1 and following with Reagent Preparation, Reverse Transcription, Amplification and Detection on day 2.*

Reagent Preparation—Part A—Area 1: PreAmplification

5.3 The Amplification Kit must be at room temperature before beginning test procedure.

Wear new gloves and a lab coat to perform the following steps:

- A.1 Determine the number of reaction tubes needed for patient specimen and control testing. Place the tubes in the MicroAmp tray and lock into place with retainer.
- A.2 Prepare the Working Master Mix: Add 100 µL of HCV Mn²⁺, v2.0 to one vial of HCV MMX, v2.0. Recap and mix well by inverting 10–15 times. *Note pink color that confirms that manganese has been added to Master Mix.* This mixture is sufficient for 12 reactions. Discard remaining Manganese Solution. *Do not vortex the working master mix.*
- A.3 Pipette 50 µL of Working Master Mix into each reaction tube with a repeat pipettor or a pipettor with an aerosol barrier or positive displacement tip. *Visually inspect the tubes for the pink color to confirm that the Working Master Mix was added.* Do not close the covers of the reaction tubes at this time.
- A.4 Place the tray and appropriate number of reaction tube caps in a resealable plastic bag, seal securely and store at 2–8°C in Area 2—Specimen Preparation Area—until ready to add samples.
- A.5 Remove and dispose of gloves. Remove labcoat and leave it in Area 1 – Reagent Preparation Area.

Important Note: Amplification must begin within 4 hours of preparing the Working Master Mix.

Specimen Preparation—Part B—Area 2: PreAmplification-Specimen Preparation Area

5.4 The Specimen Preparation and Control Kits must be at room temperature before beginning procedure.

Specimens must be at ambient temperature before use. If using frozen specimens, thaw at room temperature before use.

Use only screw-cap tubes for specimen preparation to prevent splashing and potential cross-contamination of specimens. *Do not use snap-cap tubes.*

A precipitate forms in HCV LYS, v2.0 upon storage at 2–8°C. Dissolve the precipitate by warming at 25–37°C for a maximum of 30 minutes and mixing thoroughly.

Wear new gloves and a new lab coat to perform the following steps:

- B.1 Prepare 70% ethanol: Mix 11.0 mL 95% ethanol and 4.0 mL distilled or deionized water. This mixture is sufficient for 12 tests.
- B.2 Prepare Working Lysis Reagent: Vortex HCV IC, v2.0 for 5–10 seconds. Add 100 µL HCV IC, v2.0 to one vial of HCV LYS, v2.0. Recap and mix well. Discard remaining HCV IC, v2.0.

Important Note: The Working Lysis Reagent must be must within 8 hours of preparation.

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- B.3 Label a 1.5 mL screw-cap tube for each specimen and control. Place an orientation mark on each tube.
- B.4 Vortex specimens for 3–5 seconds.
- B.5 Add 400 μ L Working Lysis Reagent to each labeled tube.
- B.6 Vortex NHP for 5–10 seconds. Add 200 μ L NHP to each control tube containing Working Lysis Reagent. Cap the tubes and vortex for 3–5 seconds.
- B.7 Vortex HCV (-) C, v2.0 and HCV (+) C, v2.0 for 5–10 seconds. Add 20 μ L of HCV (-) C, v2.0 and HCV (+) C, v2.0 to the appropriate tubes. Cap tubes and vortex for 3–5 seconds.
- B.8 Incubate specimen and control tubes for 10 minutes at 60°C \pm 2°C. Remove tubes and vortex for 10 seconds.
- B.9 Add 600 μ L 100% isopropanol to each tube. Cap tubes and vortex immediately for 3–5 seconds. Incubate all tubes for 2 minutes at room temperature.
- B.10 Place tubes in microcentrifuge with orientation mark facing outward. Centrifuge for 15 minutes at 12,500 – 16,000 x g at room temperature.
- B.11 Using a new fine tip transfer pipet for each tube, carefully aspirate and discard the supernatant without disturbing the pellet (which may not be visible).
- B.12 Add 1.0 mL 70% ethanol to each tube. Cap tubes and vortex for 3–5 seconds.
- B.13 Place tubes in microcentrifuge with the orientation mark facing outward. Centrifuge for 5 minutes at 12,500 – 16,000 x g at room temperature.
- B.14 Using a new fine tip transfer pipet for each tube, carefully aspirate and discard the supernatant without disturbing the pellet. The pellet should be clearly visible now.
- B.15 Place tubes in microcentrifuge with the orientation mark facing outward. Centrifuge at maximum speed for 3–5 seconds.
- B.16 Using a 200 μ L capacity pipettor fitted with a new tip for each tube, carefully aspirate and discard the supernatant without disturbing the pellet.
- Important Note:** Residual supernatant can inhibit the amplification.
- B.17 Add 200 μ L HCV DIL, v2.0 to each tube. Break apart the pellet with a 200 μ L pipettor fitted with an aerosol barrier tip. Cap tubes and vortex vigorously for 10 seconds. Some insoluble material may remain.
- Specimens must be amplified within 3 hours of this point. If proceeding, continue with step B.18. If not, store specimens frozen at -70°C or colder for up to one month with no more than two freeze thaws.*
- B.18 Remove MicroAmp tray from the refrigerator. Using a pipettor fitted with a new aerosol barrier or positive displacement tip for each, add 50 μ L of each processed control and specimen to the appropriate reaction tube containing Working Master Mix. Be careful to avoid transferring any precipitated material that may not have gone back into solution.
- B.19 Cap the tubes. Record positions of controls and specimens in the MicroAmp tray. Reverse transcription and amplification must be started within 45 minutes of this point.
- B.20 Transfer the MicroAmp tray with the sealed tubes to the Amplification/Detection Area.

Reverse Transcription and Amplification – Part C – Area 3: Post-Amplification/Detection Area

- 5.5 Turn on the GeneAmp PCR System 9600 or GeneAmp PCR System 2400 thermal cycler at least thirty minutes prior to beginning the amplification. Perform the following steps:
- C.1 Place the MicroAmp tray/retainer assembly into the thermal cycler block.
- C.2 Program the Applied Biosystems GeneAmp PCR System 9600 or GeneAmp PCR System 2400 thermal cycler as follows:

HOLD Program	5 minutes at 50°C
HOLD Program	30 minutes at 62°C
CYCLE Program (37 Cycles)	10 seconds at 90°C, 25 seconds at 58°C
HOLD Program	91°C (NOT TO EXCEED 3 HOURS)

Within the CYCLE programs, the ramp times and allowed setpoint error should be left at the default settings: ramp time = 0.00, setpoint error = 2°C. Link the 4 programs together into a METHOD program.

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Consult the Applied Biosystems GeneAmp PCR System 9600 or GeneAmp PCR System 2400 User's Manual for additional information on programming and operation of the thermal cycler.

C.3 Start the METHOD program. The program runs approximately one hour and 45 minutes.

C.4 Remove the tray from the thermal cycler at any time during the final HOLD program, place in the MicroAmp Base and continue immediately with step C.5.

Do not allow the reaction tubes to remain in the thermal cycler beyond the end of the final HOLD program and do not extend the final HOLD program beyond 3 hours.

DO NOT BRING AMPLIFIED SAMPLES INTO THE PRE-AMPLIFICATION AREA. AMPLIFIED CONTROLS AND SAMPLES SHOULD BE CONSIDERED A SOURCE OF POTENTIAL CONTAMINATION.

C.5 Remove the caps from the reaction tubes carefully to avoid creating aerosols of the amplification products. **Immediately** pipet 100 μ L **DN** to each column of reactions tubes using a multichannel pipettor with aerosol barrier tips, use new tips for each column, and mix by pipetting up and down.

The denatured amplicon can be held at room temperature for no more than 2 hours before proceeding to Detection (Part D). If the detection reaction can be performed within 2 hours, cap the tubes with new caps and store the denatured amplicon at 2–8°C for up to one week.

C.6 Incubate for 10 minutes at room temperature to allow complete denaturation.

Detection – Part D – Area 3: Amplification/Detection Area

5.6 Allow all reagents, the HCV MWP, v2.0 and IC MWP to warm to room temperature prior to use. Examine the 10X Wash Concentrate for any precipitate. If precipitate exists, warm to 30–37°C to dissolve.

Perform the following steps:

D.1 Prepare the Working Wash Solution: Add 1 volume 10X Wash Concentrate to 9 volumes distilled or deionized water and mix well. Store at 2–25°C, in a clean, closed plastic container. Solution is stable for two weeks.

D.2 Remove the appropriate number of 8-well MWP strips from the foil packages, 1 HCV MWP, v2.0 well and 1 IC MWP well required for each sample tested, and set into MWP frame. Return unused strips to pouch and reseal making sure the desiccant remains in the pouch.

D.3 Pipette 100 μ L HCV HYB, v2.0 to each well of the MWP.

If the denatured amplicon were stored at 2–8°C, incubate at 37°C for 2–4 minutes in order to reduce viscosity.

D.4 Using aerosol barrier tips, pipet 25 μ L of denatured amplicon to the appropriate wells of the MWP. Gently tap the plate approximately 10–15 times until the color changes from blue to light yellow. This color change indicates sufficient mixing has occurred.

D.5 Cover the MWP with the MWP lid and incubate for 1 hour at 37°C \pm 2°C.

D.6 Wash the MWP 5 times manually or by using an automated MWP washer.

For manual washing:

- a. Empty contents of plate and tap dry on paper towels.
- b. Pipet Working Wash Solution to fill each well to top (250–300 μ L). Let soak for 30 seconds. Empty contents and tap dry.
- c. Repeat step b four additional times.

For automated washing:

- a. Aspirate contents of wells.
- b. Fill each well to top with Working Wash Solution (approximately 250–300 μ L), soak for 30 seconds and aspirate dry.
- c. Repeat step b four additional times.
- d. After automated washing is complete, tap the plate dry.

D.7 Add 100 μ L AV-HRP to each well.

D.8 Cover the MWP and incubate for 15 minutes at 37°C \pm 2°C.

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D.9 Prepare Working Substrate by mixing 2.0 mL **SUB A** and 0.5 mL **SUB B** for each multiple of two, 8-well microwell plate strips (16 tests). Store at room temperature and protect from exposure to direct light.

Prepare Working Substrate no more than 3 hours before use.

D.10 Wash the MWP as described in D.6.

D.11 Add 100 μ L Working Substrate to each well.

D.12 Allow color to develop for 10 minutes at room temperature (20–25°C) in the dark.

D.13 Add 100 μ L Stop to each well.

D.14 Measure the absorbance at 450 nm within 30 minutes of adding the Stop. Record the absorbance value for each patient specimen and control tested.

6. Calculations

6.1 No calculations are performed. The results are expressed in A_{450} units. The result is compared to a predetermined instrument cutoff, also in A_{450} units.

7. Reporting Results

Reporting Format

7.1 The results generated by the AMPLICOR HCV Test, v2.0 procedure are expressed in A_{450} units and reported as positive, negative, equivocal or potentially inhibited. The cutoffs for the HCV and IC results were determined based on cumulative frequency distributions of absorbance values. These values were obtained with patient specimens, serum and EDTA plasma, tested during preclinical and clinical studies. The values are as follows:

<i>HCV A_{450} Result</i>	<i>HCV IC A_{450} Result</i>	<i>Interpretation</i>
< 0.30	≥ 0.30	Negative: HCV RNA not detected. This result does not preclude the presence of HCV RNA if specimen handling (collection, transport, processing or storage) was inadequate, interfering substances or inhibitors were present, or RNA was insufficient.
< 0.30	< 0.30	Potentially Inhibited: HCV RNA, if present, was not detectable because the specimen contained an inhibitor, or RNA was lost during specimen preparation. Inhibitors are often labile so process another aliquot of specimen and repeat test. If the same result is obtained on repeat testing, the interpretation remains Potentially Inhibited .
≥ 1.0	ANY	Positive: HCV RNA detected.
$\geq 0.30, < 1.0$	ANY	Equivocal: Inconclusive for HCV RNA. Repeat entire test procedure in duplicate, using new aliquots of specimen. When both repeat HCV A_{450} are ≥ 1.0 , final interpretation is Positive . When both repeat HCV A_{660} are < 0.30 and both HCV IC A_{450} are ≥ 0.30 , final interpretation is Negative . For any other combination of repeat test results, the interpretation is Equivocal .

7.2 Expected Values

Detection of HCV RNA, by itself, does not distinguish between acute and chronic states of infection or indicate the presence of liver disease.

A Positive result should be interpreted with caution in a patient who does not have antibody evidence of HCV infection.

A Negative result does not exclude active HCV infection.

8. Quality Control

Quality Control Information

- 8.1 *At least one replicate of the AMPLICOR HCV (-) Control and one replicate of the AMPLICOR (+) Control must be processed and included with each batch of specimens. There are no requirements regarding the position of the controls in the MicroAmp tray. In addition, the HCV Internal Control (HCV IC) must be added to each specimen and control during Specimen Preparation. After addition of HCV IC to specimens and controls, the concentration of HCV IC RNA is ≈ 400 copies per mL, which corresponds to ≈ 150 IU/mL of HCV RNA. RNA concentration in the AMPLICOR HCV (+) Control is ≈ 120 IU/mL.*
- Specimens and controls from separate specimen preparation batches may be amplified and detected at the same time. However, each separate specimen batch is validated individually by the set of controls included with the batch. Therefore, it is possible to reject one batch of specimens from a common amplification and/or detection run while accepting another batch upon the performance of the controls processed with those specimens.*
- All test specimens and controls prepared in the same batch should be amplified and detected in adjacent positions in the thermal cycler and on the detection plate. The exact order or placement of these specimens and controls in the thermal cycler or detection plate is not critical.*
- Additional controls may be tested according to guidelines or requirements of local, state and/or federal regulations or accrediting organizations.*

Control Material Preparation and Storage

- 8.2 Two controls are provided for use: AMPLICOR HCV (-) Control and AMPLICOR HCV (+) Control. The controls are liquid, ready-to-use.

Store the controls at 2–8°C. The products are stable until the expiration date. Refer to Section 5.6 for instructions on the preparation of Controls.

- 8.2 The quality control material is located in the flammable storage refrigerator (model 3551-10) along with other assay reagents in room 1-1383.

8.3

Recording Quality Control Results

- 8.4 Follow established laboratory guidelines for recording quality control results.

Acceptable Limits

- 8.5 *The HCV A_{450} of the AMPLICOR HCV (-) Control must be < 0.25 . The HCV IC A_{450} of the AMPLICOR HCV (-) Control must be ≥ 0.30 .*

The HCV A_{450} of the AMPLICOR HCV (+) Control must be ≥ 1.5 . The HCV IC A_{450} of the AMPLICOR HCV (+) Control must be ≥ 0.30 .

Corrective Actions

- 8.6 The entire run is considered to be invalid if one or both controls are not within specified limits. Repeat the entire test process: specimen and control preparation, reverse transcription, amplification and detection.
- If one or both controls are consistently outside the specified limits, contact the Roche Response Center[®] for technical assistance.*
- Specimen Processing Lysis Control*
- 8.7 *Since the AMPLICOR HCV (+) Control does not control for the lysis portion of Specimen Preparation, the user may consider a well-characterized, HCV RNA-positive specimen that is available in sufficient quantity to be included as an external control for the entire procedure. Additional external controls may be tested*

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according to guidelines or requirements of local, state and/or federal regulations or accrediting organizations.

9. Procedural Notes

Interfering Substances

9.1 Interfering substances include but are not limited to the following:

Heparin inhibits PCR; specimens collected using heparin as the anticoagulant should not be used with the AMPLICOR HCV Test, v2.0.

The effect of cryoglobulins on the AMPLICOR HCV Test, v2.0 has not been determined. Negative HCV RNA results from specimens known to contain high levels of cryoglobulins should be interpreted with caution.

The effect of elevated concentrations of alanine transferase (ALT), aspartate aminotransferase (AST), and gamma glutamyl transferase (GGT) on the AMPLICOR HCV Test, v2.0 has not been determined.

The effect of therapeutic drugs for bacterial and fungal infections on the AMPLICOR HCV Test, v2.0 has not been determined.

Limitations of the Method

9.2 Although RNA representing all recognized HCV genotypes (1–6) can be detected with this test, analytical sensitivity and other performance characteristics have not been determined for all HCV genotypes.

Detection of HCV RNA is dependent on the number of virions in the specimen and may be affected by specimen collection methods, patient factors, and/or state of infection.

The effectiveness of this test for use in screening blood, plasma, or tissue donors has not been determined.

Performance has not been determined for testing of individuals without antibody evidence of infection with HCV or for monitoring HCV-infected patients for progress of disease or response to treatment.

Procedural Precautions

9.3 *Workflow in the laboratory must proceed unidirectionally. It must begin in the Reagent Preparation area, move to the Specimen Preparation area, and then move to the Amplification/Detection area.*

9.3.1 *Reagent preparation and specimen preparation are performed in separate, segregated areas.*

9.3.2 *Supplies and equipment must be dedicated to each activity and not used for other activities or moved between areas.*

9.3.3 *Gloves must be worn in each area and must be removed before leaving that area.*

9.3.4 *Supplies, equipment, and gloves used for the preparation activities must not be used in the Amplification/Detection activities. Any amplification and detection supplies and equipment must remain in that area at all times. All pipettors, pipettes, bulbs, pipette tips, etc. must be dedicated to, and used only for, its individual PCR activity. It must not be used for any non-PCR activity.*

Procedural Limitations

9.4 *The presence of AmpErase in the Master Mix reduces the risk of amplicon contamination. However, only good laboratory practices and careful adherence to the procedures and precautions in the Method Manual may avoid contamination.*

9.5 *Only personnel with special training in PCR techniques should use this product.*

9.6 *Reliable results are dependent on adequate specimen collection, transport, storage and processing procedures.*

Technical Assistance from Manufacturer

9.5 For telephone technical assistance: Roche Response Center[®]: 1-800-526-1247

10. References

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