Laboratory Procedure Manual

Analyte: **Hepatitis C Virus RNA**

Matrix: **Serum**

Method: **AmpliPrep/COBAS® TaqMan® HCV Test**

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As performed by: **Diagnostic Reference Team (DRT)**
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Important Information for Users

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

1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

Clinical Relevance:

Hepatitis is inflammation of the liver most often caused by a virus. Viral hepatitis is a major public health problem of global importance because of the ongoing transmission of viruses that cause the disease and increased morbidity and mortality associated with the acute and chronic consequences of these infections. Global and US goals have been established for elimination of viral hepatitis as a public health threat by 2030.

In the US, the most common types of viral hepatitis are hepatitis A, B, and C. Effective vaccines are available to help prevent hepatitis A and hepatitis B. No vaccine is available for hepatitis C; however, highly effective, well-tolerated treatment can cure hepatitis C virus infection. Hepatitis D virus infection is less common in the US and can occur only among persons with hepatitis B virus infection. Hepatitis E infection also is less common in the US. These five hepatitis viruses, also called hepatitides, are well-characterized for detection with laboratory assays and are monitored in U.S. public health surveillance systems.

NHANES viral hepatitis data are used to monitor progress toward goals in *Healthy People* and the HHS *Viral Hepatitis National Strategic Plan*, which in turn support US and global viral hepatitis elimination goals. The viral hepatitis laboratory and interview components of NHANES complement data from outbreak, case-based surveillance, vital statistics, health care systems, and cohort studies that can provide timely, detailed, or longitudinal information for subnational geographic areas and disproportionately affected populations (such as persons experiencing homelessness or living in correctional facilities); however, these sources may lack information available from NHANES, such as race, ethnicity, education, income, and health status and behaviors.

Viral hepatitis data from NHANES are available beginning with the Second NHANES conducted during 1976-1980 for hepatitis A and hepatitis B, and with the Third NHANES conducted during 1988-1994 for hepatitis C, hepatitis D and hepatitis E.
Test Principle:

The hepatitis C virus (HCV) is known to be the causative agent for most blood-borne non-A, non-B hepatitis (NANBH). Studies throughout the world indicate that HCV is transmitted through contaminated blood and blood products, through blood transfusions or through other close, personal contacts. The presence of anti-HCV indicates that a person may have been infected with HCV; the presence of HCV RNA indicates that the person may be infected currently and be capable of transmitting HCV infection. In the context of NHANES, an HCV RNA positive result likely indicates chronic infection with hepatitis C virus.

HCV infection is the most common chronic blood-borne infection in the United States with an estimated seroprevalence of 1.7%. An estimated 2.4 million Americans suffer from chronic HCV infection making it the leading cause of chronic liver disease. It is estimated that >50% of those with acute hepatitis C progress to chronic infection, that can result in cirrhosis, liver cancer and

death. In the United States, chronic hepatitis C (CHC) was responsible for an estimated 14,000 deaths in 2019 and is the leading cause of liver transplantation. The burden of HCV-associated disease is likely to increase during the next 10 to 20 years as the infected cohort reaches an age at which complications of liver disease typically occur. Hepatitis C infection among younger adults has increased as a consequence of the opioid and infectious diseases syndemic, placing new generations at risk, including infants born to pregnant persons with hepatitis C infection.

In 2013, CDC revised its guidelines for Hepatitis C (HCV) testing because of 1) changes in the availability of certain commercial HCV antibody tests; 2) evidence that many persons who are identified as reactive by an HCV antibody test might not subsequently be evaluated to determine if they have current HCV infection; and 3) there have been significant advances in the development of antiviral agents with improved efficacy against HCV (Moyer, 2013). The 2020 revised guidelines recommended testing at least once for everyone aged 18 years and older, and pregnant persons during every pregnancy (Schillie, 2020).

In 2013, there was a change to the HCV testing algorithm. The flow chart for the HCV testing algorithm can be found in the laboratory method file or by following the MMWR link: https://www.cdc.gov/mmwr/pdf/wk/mm62e0507a2.pdf.

Samples found reactive to the HCV antibody screening test are tested for HCV RNA. All HCV RNA positive samples were tested for HCV genotype, and only HCV RNA negative samples were tested with an HCV antibody confirmation test.

The NHANES viral hepatitis laboratory component includes HCV antibody screening, HCV RNA testing for antibody screening reactive specimens, genotyping for HCV RNA positive specimens, and antibody confirmation for HCV RNA negative specimens.

Examined participants aged 6 years and older in the NHANES 2017-March 2020 pre-pandemic sample were eligible for the hepatitis C antibody screening, HCV RNA, HCV genotype and HCV antibody confirmation tests.

Test Principle:

Hepatitis C RNA is measured using the COBAS AmpliPrep/COBAS TaqMan, an in vitro nucleic acid amplification test for the quantitation of Hepatitis C Virus RNA in human serum or plasma on the COBAS TaqMan 48 Analyzer.

The COBAS AmpliPrep/COBAS TaqMan HCV Test is a nucleic acid amplification test for the quantitation of HCV RNA in human serum or plasma. Specimen preparation is automated using the COBAS AmpliPrep Instrument with amplification and detection automated using the COBAS TaqMan 48 Analyzer or the COBAS 5800 System.

The COBAS AmpliPrep/COBAS TaqMan HCV Test is based on three major processes: (1) specimen preparation to isolate HCV RNA; (2) reverse transcription of the target RNA to

generate complementary DNA (cDNA), and (3) simultaneous PCR amplification of target cDNA and detection of cleaved dual-labeled oligonucleotide detection probe specific to the target.

The COBAS AmpliPrep/COBAS TaqMan HCV Test permits automated specimen preparation followed by automated reverse transcription, PCR amplification and detection of HCV target RNA and HCV Quantitation Standard (QS) Armored RNA. The Master Mix reagent contains primers and probes specific for both HCV RNA and HCV QS Armored RNA. The Master Mix has been developed to ensure similar quantitation of HCV genotypes 1 through 6. The detection of amplified DNA is performed using a target-specific and a QS-specific dual-labeled oligonucleotide probe that permit independent identification of HCV amplicon and HCV QS amplicon.

The quantitation of HCV viral RNA is performed using the HCV QS. The HCV QS compensates for effects of inhibition and controls the preparation and amplification processes, allowing a more accurate quantitation of HCV RNA in each specimen. The HCV QS is a non-infectious Armored RNA construct that contains HCV sequences with identical primer binding sites as the HCV target RNA and a unique probe binding region that allows HCV QS amplicon to be distinguished from HCV target amplicon.

The HCV QS is added to each specimen at a known copy number and is carried through the specimen preparation, reverse transcription, PCR amplification and detection of cleaved dual-labeled oligonucleotide detection probes. The COBAS TaqMan Analyzer or COBAS TaqMan 48 Analyzer calculates the HCV RNA concentration in the test specimens by comparing the HCV signal to the HCV QS signal for each specimen and control.

Target Selection

Selection of the target RNA sequence for HCV depends on identification of regions within the HCV genome that show maximum sequence conservation among the various HCV genotypes. Generic silica-based specimen preparation is used to capture the HCV RNA and HCV QS RNA and defined oligonucleotides are used as primers in amplification of the HCV RNA and HCV QS RNA. A target-specific and a QS-specific dual-labeled oligonucleotide probe permit independent identification of HCV amplicon and HCV QS amplicon. Accordingly, the appropriate selection of the primers and the dual-labeled oligonucleotide probe is critical to the ability of the test to amplify and detect the HCV genotypes. The COBAS AmpliPrep/COBAS TaqMan HCV Test uses reverse transcription and PCR amplification primers that define a sequence within the highly conserved region of the 5'-untranslated region of the HCV genome. The nucleotide sequence of the primers has been optimized to yield comparable amplification of six HCV genotypes.

Specimen Preparation

The COBAS AmpliPrep/COBAS TaqMan HCV Test utilizes automated specimen preparation on the COBAS AmpliPrep Instrument by a generic silica-based capture technique. The procedure processes 850 µL of plasma or serum. The HCV virus particles are lysed by incubation at

elevated temperature with a protease and chaotropic lysis/binding buffer that releases nucleic acids and protects the released HCV RNA from RNases in serum or plasma. Protease and a known number of HCV QS RNA molecules are introduced into each specimen along with the lysis reagent and magnetic glass particles. Subsequently, the mixture is incubated and the HCV RNA and HCV QS RNA are bound to the surface of the magnetic glass particles. Unbound substances, such as salts, proteins and other cellular impurities, are removed by washing the magnetic glass particles. After separating the beads and completing the washing steps, the adsorbed nucleic acids are eluted at elevated temperature with an aqueous solution. The processed specimen, containing the magnetic glass particles as well as released HCV RNA and HCV QS RNA, is added to the amplification mixture and transferred to the COBAS TaqMan Analyzer or COBAS TaqMan 48 Analyzer. The HCV target RNA and the HCV QS RNA are then reverse transcribed, amplified and simultaneously detected by cleavage of a target-specific and a QS-specific dual-labeled oligonucleotide probe.

Reverse Transcription and PCR Amplification

The reverse transcription and PCR amplification reaction is performed with the thermostable recombinant enzyme *Thermus specie* DNA Polymerase (Z05). In the presence of manganese (Mn^{2+}) and under the appropriate buffer conditions, Z05 has both reverse transcriptase and DNA polymerase activity. This allows both reverse transcription and PCR amplification to occur together with real-time detection of the amplicon.

Processed specimens are added to the amplification mixture in amplification tubes (Ktubes) in which both reverse transcription and PCR amplification occur. The reaction mixture is heated to allow a downstream primer to anneal specifically to the HCV target RNA and to the HCV QS RNA. In the presence of Mn^{2+} and excess deoxynucleotide triphosphates (dNTPs), including deoxyadenosine, deoxyguanosine, deoxycytidine and deoxyuridine triphosphates, Z05 polymerase extends the annealed primers forming a DNA strand complementary to the RNA target.

Target Amplification

Following reverse transcription of the HCV target RNA and the HCV QS RNA, the Thermal Cycler in the COBAS TaqMan 48 Analyzer heats the reaction mixture to denature the RNA:cDNA hybrid and to expose the specific primer target sequences. As the mixture cools, the primers anneal to the target DNA. The thermostable *Thermus specie* Z05 DNA Polymerase (Z05) in the presence of Mn^{2+} and excess deoxynucleotide triphosphates (dNTPs), including deoxyadenosine, deoxyguanosine, deoxycytidine and deoxyuridine (in place of thymidine) triphosphates, extends the annealed primers along the target template to produce a doublestranded DNA molecule termed an amplicon. The COBAS TaqMan Analyzer or COBAS TaqMan 48 Analyzer automatically repeats this process for a designated number of cycles, with each cycle intended to double the amount of amplicon DNA. The required number of cycles is preprogrammed into the COBAS TaqMan Analyzer or COBAS TaqMan 48 Analyzer.

Amplification occurs only in the region of the HCV genome between the primers; the entire HCV genome is not amplified.

Selective Amplification

Selective amplification of target nucleic acid from the specimen is achieved in the COBAS AmpliPrep/COBAS TaqMan HCV Test by the use of AmpErase (uracil-Nglycosylase) enzyme and deoxyuridine triphosphate (dUTP). The AmpErase enzyme recognizes and catalyzes the destruction of DNA strands containing deoxyuridine but not DNA containing deoxythymidine. Deoxyuridine is not present in naturally occurring DNA, but is always present in amplicon due to the use of deoxyuridine triphosphate as one of the dNTPs in the Master Mix reagent; therefore, only amplicon contains deoxyuridine. Deoxyuridine renders contaminating amplicon susceptible to destruction by the AmpErase enzyme prior to amplification of the target DNA. Also, any nonspecific product formed after initial activation of the Master Mix by manganese is destroyed by the AmpErase enzyme. The AmpErase enzyme, 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, the amplicon DNA chain breaks at the position of the deoxyuridine, thereby rendering the DNA non-amplifiable. The AmpErase enzyme remains inactive for a prolonged period of time once exposed to temperatures above 55ºC, i.e., throughout the thermal cycling steps, and therefore does not destroy target amplicon formed after PCR reaction.

Detection of PCR Products in a COBAS TaqMan Test

The COBAS AmpliPrep/COBAS TaqMan HCV Test utilizes real-time PCR technology. The use of dual-labeled fluorescent probes allows for real-time detection of PCR product accumulation by monitoring of the emission intensity of fluorescent reporter dyes released during the amplification process. The probes consist of HCV and HCV QS-specific oligonucleotide probes with a reporter dye and a quencher dye. In the COBAS AmpliPrep/ COBAS TaqMan HCV Test, the HCV and HCV QS probes are labeled with different fluorescent reporter dyes. When these probes are intact, the fluorescence of the reporter dye is suppressed by the proximity of the quencher dye due to Förster-type energy transfer effects. During PCR, the probe hybridizes to a target sequence and is cleaved by the 5' → 3' nuclease activity of the thermostable Z05 DNA polymerase. Once the reporter and quencher dyes are released and separated, quenching no longer occurs, and the fluorescent activity of the reporter dye is increased. The amplification of HCV RNA and HCV QS RNA are measured independently at different wavelengths. This process is repeated for a designated number of cycles, each cycle effectively increasing the emission intensity of the individual reporter dyes, permitting independent identification of HCV RNA and HCV QS RNA. The PCR cycle where a growth curve starts exponential growth is related to the amount of starting material at the beginning of the PCR.

Fundamentals of COBAS TaqMan Test Quantitation

The COBAS AmpliPrep/COBAS TaqMan HCV Test is inherently quantitative over a wide dynamic range since the monitoring of amplicon is performed during the exponential phase of amplification. The higher the HCV titer of a specimen, the earlier the fluorescence of the reporter dye of the HCV probe rises above the baseline fluorescence level. Since the amount of HCV QS RNA is constant between all specimens, the fluorescence of the reporter dye of the HCV QS probe should appear at the same cycle for all specimens. In specimens, where the QS amplification and detection is affected by inhibition or poor specimen recovery, the appearance of fluorescence will be delayed, thereby enabling the calculated titer of HCV target RNA to be adjusted accordingly. The appearance of the specific fluorescent signals is reported as a critical threshold value (Ct). The Ct is defined as the fractional cycle number where reporter dye fluorescence exceeds a predetermined threshold (the Assigned Fluorescence Level), and starts the beginning of an exponential growth phase of this signal. A higher Ct value indicates a lower titer of initial HCV target material. A 2-fold increase in titer correlates with a decrease of 1 Ct for target HCV RNA; a 10-fold increase in titer correlates with a decrease of 3.3 Ct.

HCV RNA Quantitation

The COBAS AmpliPrep/COBAS TaqMan HCV Test quantitates HCV viral RNA by utilizing a second target sequence (HCV Quantitation Standard) that is added to each test specimen at a known concentration. The HCV QS is a non-infectious Armored RNA construct, containing fragments of HCV sequences with primer binding regions identical to those of the HCV target sequence. The HCV QS generates an amplification product of the same length and base composition as the HCV target RNA. The detection probe binding region of the HCV QS has been modified to differentiate HCV QS amplicon from HCV target amplicon.

During the annealing phase of the PCR on the COBAS TaqMan Analyzer or COBAS TaqMan 48 Analyzer, the specimens are illuminated and excited by filtered light and filtered emission fluorescence data are collected for each specimen. The readings from each specimen are then corrected for instrumental fluctuations. These fluorescence readings are sent by the instrument to the AMPLILINK software and stored in a database. Pre-Checks are used to determine if the HCV RNA and HCV QS RNA data represent sets that are valid, and flags are generated when the data lie outside the preset limits. After all Pre-Checks are completed and passed, the fluorescence readings are processed to generate Ct values for the HCV RNA and the HCV QS RNA. The lot-specific calibration constants provided with the COBAS AmpliPrep/COBAS TaqMan HCV Test are used to calculate the titer value for the specimens and controls based upon the HCV RNA and HCV QS RNA Ct values. The COBAS AmpliPrep/COBAS TaqMan HCV Test is standardized against the First WHO International Standard for Hepatitis C Virus RNA for Nucleic Acid Amplification Technology Assays (NIBSC code 96/790) and titer results are reported in International Units (IU/mL).

2. SAFETY PRECAUTIONS

Test kits 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. Controls and samples should be handled as if infectious using safe laboratory procedures such as those outlined in *Biosafety in Microbiological and Biomedical Laboratories* and in the CLSI Document M29-A.

Observe universal precautions when performing the assay, thoroughly clean and disinfect all work surfaces with a freshly prepared solution of 0.5% sodium hypochlorite in deionized or distilled water, handle samples with extreme care to prevent sample contamination, ***use*** new, sterile aerosol barrier or positive displacement RNase-free pipette tips and sterile pipettes, wear personal protective apparel, disposable gloves and eyewear during all steps of this method to minimize both infectious and chemical contamination hazards.

Do not eat, drink, smoke, or apply cosmetics in areas where reagents or samples are handled. If skin or mucous membrane exposure occurs, immediately wash the area with copious amounts of water. Seek medical advice. Place all plastic and glassware contaminated with serum in a plastic autoclave bag for disposal. Do not use components beyond the expiration date on the kit. Alterations in the physical appearance of kit components may indicate instability or deterioration. Do not pool reagents from different lots or from different bottles of the same lot. Do not mix reagent cassettes or controls from different kits. Do not open COBAS AmpliPrep cassettes and exchange, mix, remove or add bottles. Store the kit away from any source of contaminating DNA, especially amplified nucleic acid. We recommend Biosafety Level 2 containment procedures as described in CDC/NIH publication #93-8395[i] be used by those handling test specimens and kit reagents. Use a Unidirectional work flow proceeding from the sample preparation to the amplification and detection steps. To help prevent laboratory areas from becoming contaminated with amplified RT-PCR product, maximize the physical separation of the pre- and post-amplification steps. Do not return samples, equipment, or reagents to the area where you performed the previous step. If you need to return to a previous work area, first perform the appropriate anti-contamination safeguards. Avoid microbial and RBase contamination of reagents.

Use all pipetting devices and instruments with care and follow the manufacturer's instructions for calibration and quality control.

HCV QS, CAP/CTM Mn and **HCV MMX** contain sodium azide. Sodium azide may react with lead and copper plumbing to form highly explosive metal azides. While disposing of sodium azide containing solutions down laboratory sinks, flush the drains with a large volume of water to prevent azide buildup.

Do not allow **HCV CS2** and liquid waste from the COBAS AmpliPrep Instrument, which contain guanidine thiocyanate, to contact sodium hypochlorite (bleach) solution. These mixtures can produce a highly toxic gas.

When disposing of used COBAS AmpliPrep Sample Processing Units (SPUs), which contain guanidine thiocyanate, avoid any contact with sodium hypochlorite (bleach) solution. These mixtures can produce a highly toxic gas.

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

Data Management System (DMS) was used through December 31, 2019.
The run information can be uploaded into the computerized database (DMS) after the run information is extracted by software. This database was custom-designed for the management of CDC Division of Viral Hepatitis (DVH) Laboratory Branch (LB) test results, and functions within SQL Server software (Microsoft, Redmond, WA) with a .NET (Microsoft, Redmond, WA) user interface. In August 2019, laboratory data management was transferred to the CDC Enterprise Laboratory Information System (ELIMS), where NHANES functionality was reproduced and improved over time to include more process automation. DMS was maintained in parallel through December 31, 2019, when it was discontinued. Finished DMS data were reviewed by the laboratory supervisor and transmitted to the NCHS along with other NHANES data. Files stored on the CDC Local Area Network (LAN) were automatically backed up nightly by CDC Data Center staff.
Documentation for data system maintenance was maintained with printed copies of data records for 2 years.

CDC Enterprise Laboratory Information System (ELIMS) is currently used since January 1, 2020, for accessioning, test results processing, reporting and storage. Finished ELIMS data are reviewed by the laboratory supervisor and transmitted to the NCHS along with other NHANES data. All information about the accessioned specimens, traceability of the diagnostic process, test runs and reported results are stored in ELIMS database, are archived after 12 months and can be retrieved any time upon request.

All necessary information about the ELIMS at CDC can be found on an intranet site accessible by laboratory staff.

Other information in the database may typically include the CDC sample identification number (CSID), CDC Unique ID (CUID), the CDC Local ID (NHANES specimen ID), the date collected, the date tested and results of testing for other hepatitis markers.

Both DMS-generated and ELIMS-generated NHANES reports are reviewed by the laboratory Technical Supervisor and transmitted to the CDC Division of Viral Hepatitis Epidemiology and Surveillance Branch for secondary QC and subsequent submission to NCHS along with other NHANES data.

4. SPECIMEN COLLECTION, STORAGE, AND HANDLING PROCEDURES; CRITERIA FOR SPECIMEN REJECTION

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

No special patient preparation before collection is necessary.

Blood should be collected in sterile collection tubes using red tops.

Collect one red top tube using standard venipuncture techniques. Follow the manufacturer's instructions for use of the collection tubes.

Separate serum from whole blood within 6 hours of collection by centrifugation at 1500 x g for 20 minutes at room temperature.

Transfer serum to a properly identified, sterile, screw-cap, polypropylene tube after centrifugation.

Serum specimens may be stored at 2-8°C (36–46 °F) for up to 72 hours or frozen at <-20°C (-4 °F) or colder for up to 6 weeks. Serum specimens may be frozen and thawed up to five times without a loss of HCV RNA.
Specimens and controls should be handled as if infectious using safe laboratory procedures such as those outlined in *Biosafety in Microbiological and Biomedical Laboratories* and in the CLSI Document M29-A. Thoroughly clean and disinfect all work surfaces with a freshly prepared solution of 0.5% sodium hypochlorite in deionized or distilled water.

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

Not applicable for this procedure.

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

a. Instrumentation and Software

COBAS AmpliPrep Instrument

COBAS AmpliPrep TaqMan 48 Analyzer

AMPLILINK Software, Version 3.3.6 Series

Data Station for the AMPLILINK software, with printer

Multichannel pipettor (capacity 25 and 100 μL)

Aerosol barrier or positive displacement RNase-free tips (25 and 100 μL) and barrier-free tips

b. Reagents

Reagent Labeling and Preparation
All reagents are labeled by the manufacturer. The labeling includes contents, lot number, expiration date, and storage instructions. All reagents are liquid, ready-to-use.

Reagent Storage and Use

Do not freeze reagents or controls.

Store **HCV CS1, HCV CS2, HCV CS3** and **HCV CS4** at 2-8ºC (36–46 °F). Unused, these reagents are stable until the expiration date indicated. Once used, these reagents are stable for 28 days at 28°C (82°F) or until the expiration date, whichever comes first. **HCV CS1, HCV CS2, HCV CS3** and **HCV CS4** can be used for a maximum of 4 instrument cycles, up to a maximum of 64 hours cumulative on board the COBAS AmpliPrep Instrument. Reagents must be stored at 2-8°C (36–46 °F) between instrument cycles.

Store **HCV H(+)C, HCV L(+)C** and **CTM (–) C** at 2-8ºC (36–46 °F). The controls are stable until the expiration date indicated. Once opened, any unused portion must be discarded.

Store Barcode clips [**HCV H(+)C Clip, HCV L(+)C Clip** and **HCV (–) C Clip**] at 2- 30°C (36–86 °F).

Store **PG WR** at 2-30°C (36–86 °F). **PG WR** is stable until the expiration date indicated. Once opened, this reagent is stable for 28 days at 2-30°C (36–86 °F) or until the expiration date, whichever comes first.

Do not pool reagents from different lots or from different bottles of the same lot. Do not mix reagent cassettes or controls from different kits. Do not open COBAS AmpliPrep cassettes and

exchange, mix, remove or add bottles. Do not pool reagents. Dispose of unused reagents and waste in accordance with all local, country, state, and federal regulations.

Handle all reagents with caution and avoid contact with skin, eyes, or mouth. Refer to the package insert for any known toxicity.

HCV QS, CAP/CTM Mn^{2+} and **HCV MMX** contain sodium azide. Sodium azide may react with lead and copper plumbing to form highly explosive metal azides. While disposing of sodium azide containing solutions down laboratory sinks, flush the drains with a large volume of water to prevent azide buildup.

Do not allow **HCV CS2** and liquid waste from the COBAS AmpliPrep Instrument, which contain guanidine thiocyanate, to contact sodium hypochlorite (bleach) solution. These mixtures can produce a highly toxic gas.

When disposing of used COBAS AmpliPrep Sample Processing Units (SPUs), which contain guanidine thiocyanate, avoid any contact with sodium hypochlorite (bleach) solution. These mixtures can produce a highly toxic gas.

These reagents carry a highly flammable warning: HCV CS1.

These reagents are marked as **harmful or irritants**: HCV CS1, HCV CS2, and HCV CS3.

7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

a. **Calibration Curve**

No calibration curve is produced. **AMPLILINK Software:**
Determines the Cycle Threshold value (Ct) for the HCV RNA and the HCV QS RNA.

Determines the HCV RNA concentration based upon the Ct values for the HCV RNA and HCV QS RNA and the lot-specific calibration coefficients provided on the cassette barcodes.

Determines that the calculated IU/mL for **HCV L(+)C** and **HCV H(+)C** fall within the lot specific assigned ranges encoded on the COBAS AmpliPrep/COBAS TaqMan HCV Test reagent cassette barcodes supplied with the kit.

b. **Verification**

Not Applicable

8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

a. **Preliminaries**

Sample Preparation
If using frozen specimens, place the specimens at room temperature until completely thawed and vortex for 3-5 seconds before use. Controls should be removed from 28°C (82°F) storage and equilibrated to ambient temperature before use.

b. **Instrument Setup**

Operation of Assay Procedure

Run Size and Workflow
Each kit contains reagents sufficient for 48 tests, which may be performed in batches of 12 to 24 tests. At least one replicate each of **CTM (–) C, HCV L(+)C** and **HCV H(+)C** must be included in each batch (*see* "Quality Control" section).

Workflow

The COBAS TaqMan 48 Analyzer run must be started within 120 minutes following completion of specimen and control preparation.

Note: Do not freeze or store processed specimens and controls at 2-8°C (36–46 °F).

Part A. Maintenance and Priming
A1. The COBAS AmpliPrep Instrument is ready for operation in stand-by mode.

A2. Turn the Data Station for the AMPLILINK software **ON**. Prepare the Data Station as follows:

a. Log onto Windows XP.
b. Double click the AMPLILINK software icon.
c. Log onto AMPLILINK software by entering the assigned User ID and password.

A3. Check the supply of **PG WR** using the **Status** Screen and replace if necessary.

A4. Perform all Maintenance that is listed in the Due Tab. The COBAS AmpliPrep Instrument will automatically prime the system.

Part B. Loading of Reagent Cassettes
Note: All reagent cassettes should be removed from 2-8°C (36–46 °F) storage, immediately loaded onto the COBAS AmpliPrep Instrument and allowed to equilibrate to ambient temperature on the instrument for at least 30 minutes before the first specimen is to be

processed. Do not let reagent cassettes come to ambient temperature outside the instrument as condensation may form on the barcode labels. Do not wipe off condensation if it appears on the barcode labels.

B1. Place **HCV CS1** onto a reagent rack. Place **HCV CS2, HCV CS3** and **HCV CS4** onto a separate reagent rack.

B2. Load the reagent rack containing **HCV CS1** onto rack position **A** of the COBAS® AmpliPrep Instrument.

B3. Load the reagent rack containing **HCV CS2, HCV CS3** and **HCV CS4** onto rack position **B, C, D** or **E** of the COBAS® AmpliPrep Instrument (*see* Table 1 for additional information).

Part C. Loading of Disposables

Note: Determine the number of COBAS AmpliPrep® reagent cassettes, Sample Processing Units (SPUs), Input Sample tubes (S-tubes), K-tips and K-tubes needed. One SPU, one Input S-tube, one K-tip and one K-tube are needed for each specimen or control.

Multiple configurations for use of the COBAS AmpliPrep Instrument with the COBAS TaqMan Analyzer or COBAS TaqMan 48 Analyzer are possible. For reference, *see* Table 1 below. Depending on the configuration used, load the appropriate number of reagent cassette racks, sample racks with Input S-tubes, SPU racks, K-tip racks, Ktube racks and K-carriers on K-carrier racks onto the respective rack positions of the COBAS AmpliPrep Instrument (*see* Table 1 for additional information).

C1. Place the SPUs in the SPU rack(s) and load the rack(s) onto rack position **J, K** or **L** of the COBAS® AmpliPrep Instrument.

C2. Depending on the configuration used, load full K-tube rack(s) onto rack position **M, N, O** or **P** of the COBAS® AmpliPrep Instrument.

C3. Load full K-tip rack(s) onto rack position **M, N, O** or **P** of the COBAS® AmpliPrep Instrument.

C4. For configurations 3 to 5 using the COBAS® TaqMan® 48 Analyzer, load Kcarriers on Kcarrier rack(s) onto rack position **M, N, O** or **P** of the COBAS® AmpliPrep Instrument

Table 1. Possible Configurations for using COBAS AmpliPrep Instrument with COBAS TaqMan Analyzer or COBAS TaqMan 48 Analyzer

Configuration		Transfer Mode to COBAS TaqMan Analyzer or COBAS TaqMan® 48 Analyzer	Racks, Carriers, and Disposables	Position on COBAS AmpliPrep Instrument
3, 4	COBAS AmpliPrep Instrument plus 1 – 2 COBAS TaqMan 48 Analyzer(s)	Manual transfer of K-carrier via Kcarrier rack(s) onto COBAS TaqMan 48 Analyzer	K-tubes are placed on the sample rack	F-H
			K-tips in full K-tip racks	M-P
			Input S-tubes on sample racks	F-H
			SPUs in SPU rack	J-L
			CS1 on Cassette rack	A
			CS2, CS3, CS4 on Cassette rack	B-E
			Empty barcoded K-carrier on K-carrier rack	M-P
			After specimen processing is finished: Ktubes in K-carrier on Kcarrier rack	Same as above (M-P)

Part D. Ordering and Loading of Specimens

D1. Prepare sample racks as follows: Attach a barcode label clip to each sample rack position where a specimen (S-tube) is to be placed. Attach one of the specific barcode label clips for the controls [**CTM (–) C, HCV L(+)C** and **HCV H(+)C**] to each sample rack position where the controls (S-tube) are to be placed. The barcode label clips for controls should have the same control lot number as the lot number on the control vials in the kit. Take care in assigning the right control to the position with the appropriate control barcode clip. Place one Input S-tube into each position containing a barcode label clip.

D2. Using the AMPLILINK software, create specimen orders for each specimen and control in the **Orders** window **Sample** folder. Select the appropriate test file and complete by saving.

D3. Assign specimen and control orders to sample rack positions in the **Orders** window **Sample Rack** folder. The sample rack number must be for the rack prepared in Step D1.

D4. Print the **Sample Rack Order** report to use as a worksheet.

D5. Prepare specimen and control racks in the designated area for specimen and control addition as follows: Vortex each specimen and control [**CTM (–) C, HCV L(+)C** and **HCV H(+)C**] for 3 to 5 seconds. Avoid contaminating gloves when manipulating the specimens and controls.

D6. Transfer 1000 to 1050 µL of each specimen and control [**CTM (–) C, HCV L(+)C** and **HCV H(+)C**] to the appropriate barcode labeled Input S-tube using a micropipettor with an aerosol barrier or positive displacement RNase-free tip. ***Avoid transferring particulates and/or fibrin clots from the original specimen to the Input S-tube.***
Specimens and controls should be transferred to tube positions as assigned and recorded on the worksheet in step D4. The barcode label clips for controls should have the same control lot number as the lot number on the control vials in the kit. Assign the right control to the position with the appropriate control barcode clip. ***Avoid contaminating the upper part of the S-tubes with specimens or controls.***

D7. For configurations 1 and 2, load the sample rack(s) filled with Input S-tubes onto rack positions **F, G** or **H** of the COBAS AmpliPrep Instrument.

D8. For configurations 3 to 5 using the COBAS TaqMan 48 Analyzer, load sample rack(s) with Input S-tubes and K-tubes (one for each Input S-tube, loaded in the right position adjacent to Input S-tubes) onto rack position **F, G** or **H** of the COBAS AmpliPrep Instrument.

Part E. Start of COBAS AmpliPrep Instrument Run
E1. Start the COBAS AmpliPrep Instrument using the AMPLILINK software.

Part F. End of COBAS AmpliPrep Instrument Run and Transfer to COBAS TaqMan Analyzer or COBAS TaqMan 48 Analyzer (Only for Configurations 2–5)

F1. Check for flags or error messages in the system screen.

F2. Remove processed specimens and controls from the COBAS AmpliPrep Instrument on either sample racks (for COBAS TaqMan Analyzer without Docking Station) or Kcarrier racks (for COBAS TaqMan 48 Analyzer), depending on the configuration (for further details see Part G).

F3. Remove waste from the COBAS AmpliPrep Instrument.
Note: All processed specimens and controls should not be exposed to light after completion of specimen and control preparation.

Amplification and Detection

COBAS TaqMan Analyzer or COBAS TaqMan 48 Analyzer Set-up

The COBAS TaqMan Analyzer or COBAS TaqMan 48 Analyzer run must be started within 120 minutes following completion of specimen and control preparation. ***Note: Do not freeze or store processed specimens and controls at 2-8°C (36–46 °F)***.

Part G. Loading Processed Specimens

G1. Depending on the instrument configuration, perform the appropriate steps to transfer the Ktubes to the COBAS TaqMan Analyzer or COBAS TaqMan 48 Analyzer: *Configuration 1:* Automated transfer of K-carrier via docking station to COBAS TaqMan Analyzer. Manual intervention is unnecessary.
Configuration 2 and 5: Manual transfer of K-tubes in sample rack(s) to COBAS TaqMan Analyzer
Configuration 3, 4 and 5: Manual transfer of K-carrier on K-carrier rack(s) to the COBAS

TaqMan 48 Analyzer. Manual transfer of K-carriers into COBAS TaqMan 48 Analyzer using the K-carrier Transporter.

Part H. Start of COBAS TaqMan 48 Analyzer Run
H1. Start the COBAS TaqMan 48 Analyzer by one of the options below depending on the configuration used:

Configuration 1: No intervention necessary.

*Configuration 2 and 5:*Automatic start of the COBAS TaqMan Analyzer after insertion of sample rack(s).

Configuration 3, 4 and 5: Fill K-carrier with empty K-tubes if fewer than 6 K-tubes on the K-carrier. Filling is guided by the AMPLILINK software. Open thermal cycler cover, load Kcarrier into thermal cycler and close lid. Start the COBAS TaqMan 48 Analyzer run.

Part I. End of COBAS TaqMan 48 Analyzer Run

I1. At the completion of the COBAS TaqMan 48 Analyzer run, print Results Report. Check for flags or error messages in the Result section. Specimens with flags and comments are interpreted as described in the Results section. After acceptance, store data in archive.

I2. Remove used K-tubes from the COBAS TaqMan 48 Analyzer.

c. ***Reporting results***

The COBAS TaqMan 48 Analyzer automatically determines the HCV RNA concentration for the specimens and controls. The HCV RNA concentration is expressed in International Units (IU)/mL.

AMPLILINK Software:

- Determines the Cycle Threshold value (Ct) for the HCV RNA and the HCV QS RNA.
- Determines the HCV RNA concentration based upon the Ct values for the HCV RNA and HCV QS RNA and the lot-specific calibration coefficients provided on the cassette barcodes.
- Determines that the calculated IU/mL for **HCV L(+)C** and **HCV H(+)C** fall within the lot specific assigned ranges encoded on the COBAS AmpliPrep/COBAS TaqMan HCV Test reagent cassette barcodes supplied with the kit.

Results are interpreted as follows:

Titer Result	**Interpretation**
Target Not Detected	No Ct value for HCV obtained. Report results as "HCV RNA not detected".
<4.30E+01 IU/mL	Below 4.30E+01 IU/mL (lower limit of quantitation, LLoQ); HCV RNA is not quantifiable.
≥4.30E+01 IU/mL and ≤6.90E+07 IU/mL	Results greater than or equal to 43 IU/mL and less than or equal to 6.90E+07 IU/mL are within the Linear Range of the assay.
> 6.90E+07 IU/mL	Results are above the range of the assay. Report results as "greater than 6.90E+07 HCV RNA IU/mL". If quantitative results are desired, the original specimen should be diluted 1:100 with HCV-negative human serum or EDTA plasma, depending on the matrix of the original specimen, and the test repeated. Multiply the reported result by the dilution factor.

d. Recording of Data

The Analyzer automatically determines the HCV RNA titer for the sample or control.

If **Negative Control, HCV Low Positive Control or HCV High Positive Control** is invalid then the entire run is invalid, repeat the entire run including control and sample preparation, reverse transcription, amplification and detection.

A valid run may include both valid and invalid sample results depending on whether flags and/or comments are obtained for the individual samples.

e. Calculations

Not Applicable

f. Special Procedure Notes

Workflow in the laboratory must proceed unidirectional. It must begin in the Reagent Preparation area, move to the Specimen Preparation area, and then move to the Amplification/Detection area. Reagent preparation and specimen preparation are performed in separate, segregated areas.

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

Gloves must be worn in each area and must be removed before leaving that area. 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 nonPCR activity.

9. REPORTABLE RANGE OF RESULTS

Calculated results greater than or equal to 4.30 E+01 IU/mL and less than or equal to 6.90 E+07 IU/mL are within the linear range of the assay. For NHANES results are reported as positive or negative (i.e., no Ct value for HCV obtained); quantitative values are not reported.

10. QUALITY CONTROL (QC) PROCEDURES

One replicate each of the COBAS TaqMan Negative Control, the HCV Low Positive Control and the HCV High Positive Control must be included in each test batch. The batch is valid if no flags appear for any of the controls [**HCV L(+)C, HCV H(+)C** and **CTM (–) C**]. Based on results of a carry-over contamination study with alternating high positive HCV samples and HCV negative samples, there are no requirements regarding the position of the controls on the sample rack. Check the batch printout for flags and comments to ensure that the batch is valid.

Negative Control
The **CTM (–) C** must yield a "Target Not Detected" result. If the **CTM (–) C** is flagged as invalid, then the entire batch is invalid. Repeat the entire process (specimen and control preparation,

amplification and detection). If **CTM (–) C** is consistently invalid in multiple batches, contact your local Roche office for technical assistance.

Positive Controls The assigned range for **HCV L(+)C** and **HCV H(+)C** is specific for each lot of reagents, and is provided on the COBAS AmpliPrep/COBAS TaqMan HCV Test reagent cassette barcodes.

The HCV RNA IU/mL for **HCV L(+)C** and **HCV H(+)C** should fall within their assigned ranges. If one or both of the positive controls are flagged as invalid, then the entire batch is invalid. Repeat the entire process (specimen and control preparation, amplification and detection). If the HCV RNA titer of one or both of the positive controls is consistently outside the assigned ranges in multiple batches, contact your local Roche office for technical assistance.

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
Three controls are provided for use: HCV L(+)C, HCV H(+)C and CTM (–) C . The controls are liquid, ready-to-use. *Store the controls at 2–8°C (36–46 °F). The products are stable until the expiration date.*

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

The entire run is considered to be invalid if one or more controls are not within specified limits.

Repeat the entire test process: specimen and control preparation, reverse transcription, amplification and detection.

If one or more controls are consistently outside the specified limits, contact the Roche Response Center for technical assistance.

Specimen Processing Lysis Control: Since the positive controls do 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.

12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS

Interfering Substances

Interfering substances include but are not limited to the following:

Elevated levels of triglycerides, bilirubin, albumin, hemoglobin and human DNA in specimens as well as the presence of autoimmune diseases such as Systemic Lupus Erythematosus (SLE), Rheumatoid Arthritis (RA), and Antinuclear Antibody (ANA) have been shown not to interfere with the quantitation of HCV RNA by the COBAS AmpliPrep/COBAS TaqMan HCV Test.

The following drug compounds tested at the Peak Plasma Level (Cmax) and at 3 times the Cmax have been shown not to interfere with the quantitation of HCV RNA by the

COBAS AmpliPrep/COBAS TaqMan HCV Test:
HIV Protease Inhibitors Indinavir Saquinavir Ritonavir Nelfinavir Amprenavir Lopinavir/Ritonavir
Nucleoside HIV Reverse Transcriptase Inhibitors and DNA Polymerase Inhibitors Lamivudine Zidovudine Stavudine Abacavir Didanosine
Non-nucleoside HIV Reverse Transcriptase Inhibitors Nevirapine Efavirenz **HIV Fusion Inhibitors** Enfurvitide
Immune Modulators Interferon alfa-2a Interferon alfa-2b Peginterferon alfa-2a Peginterferon alfa-2a + Ribavirin Interferon alfa-2b+ Ribavirin
Antidepressants Paroxetine HCl Fluoxetine Sertraline
Compounds for Treatment of Herpes Viruses Ganciclovir Valganciclovir Acyclovir

Limitations of the Method

As with any test procedure, good laboratory technique is essential to the proper performance of this assay.

Procedural Limitations

This test has been validated for use with only human serum or plasma collected in EDTA anticoagulant. Testing of other specimen types may result in inaccurate results.

Quantitation of HCV RNA is dependent on the number of virus particles present in the specimen and may be affected by specimen collection methods, patient factors (e.g., age, presence of symptoms) and stage of infection.

Though rare, mutations within the highly conserved regions of the viral genome covered by the COBAS AmpliPrep/COBAS TaqMan HCV Test primers and/or probe may result in the underquantitation of or failure to detect the presence of the virus in this circumstance.

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

The presence of AmpErase enzyme in the COBAS AmpliPrep/COBAS TaqMan HCV Master Mix reduces the risk of amplicon contamination. However, contamination from HCV positive controls and clinical specimens can be avoided only by good laboratory practices and careful adherence to the procedures specified in this Package Insert.

Use of this product should be limited to personnel trained in the techniques of PCR.

This product can only be used with the COBAS AmpliPrep Instrument and the COBAS® TaqMan Analyzer or COBAS TaqMan 48 Analyzer.

If another assay was initially used for quantitation of HCV viral RNA in order to assess treatment effect on the patient, it is recommended that prior to switching to the COBAS

AmpliPrep/COBAS TaqMan HCV Test users perform method correlation studies in their laboratory to quantify assay differences.

13. REFERENCE RANGES (NORMAL VALUES

All normal, noninfected humans should have negative values for HCV RNA.

14. CRITICAL CALL RESULTS ("PANIC VALUES")

Not applicable.

15. SPECIMEN STORAGE AND HANDLING DURING TESTING

Specimens may remain at 15°C-25°C (59-77°F) during preparation and testing only.

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

Other FDA-licensed tests for HCV RNA 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) for specimen tracking.

Specimens in long-term storage are arranged by study group. The storage location of each sample is listed with the test data. For NHANES, residual specimens are stored frozen at -60^{o}C (-76 ^{o}F) or colder and returned to the NCHS specimen bank after testing for each cycle has been completed.

19. SUMMARY STATISTICS AND QC GRAPHS

Qualitative assays are assays with a positive, negative or borderline/indeterminate result. The absorbance or reactivity values of specimens are compared with a cutoff value that is a ratio of the negative control mean and the positive control mean. Since the controls are read as cutoff values, plots of these values are not generated for quality control purposes.

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