

HALT-C Virology Lab Procedures

I. Specimen Criteria

Acceptable criteria:

Serum is acceptable for HCV RNA qualitative and quantitative assays and HCV RNA genotype assay. Hemolysed, lipemic or icteric specimens do not affect these assays; therefore, these conditions are acceptable.

Preparation of Blood Samples for Testing:

1. Process the specimens within 2-4 hours of draw.
2. When specimens arrive for processing, make sure the specimens have been allowed to clot at room temperature for approximately 30 minutes.
3. Centrifuge the vacutainer tubes @ 2,500 – 3,500 rpm for 10-15 minutes.
4. Aliquot 1 ml of serum into aliquot tubes. 2 ml Sarstedt tubes (Sarstedt, sterile color top tubes, 2 ml cat #72.694.996) are preferred by the UW Viral Hepatitis Laboratory. If they are not available, please call the lab at 206-731-3461.
5. Place appropriate labels on each of the aliquot tubes and immediately place them in a -20°C or -70°C freezer until ready to ship.

II. Specimen Shipping and Receiving

Specimen Shipping:

1. Allowable days of shipping to UW are Mondays, Tuesdays, and Wednesdays unless other arrangements have been made. Thursdays are acceptable as long as all parties have been notified.
2. Specimens should be sent in either 1.5 ml Sarstedt tubes (cat. #72.694.006) or 2.0ml Sarstedt tubes (cat. #72.694.996). If these tubes are not available, call the UW Viral Hepatitis lab at (206) 731-3461.
3. Specimens should be sent via Federal Express or an alternate source overnight on dry ice.
4. There should be sufficient dry ice in the box to last approximately 3 days.
5. Fill out appropriate shipping forms for sending biohazardous specimens.
6. The day the specimens are sent to the UW Viral Hepatitis lab, notify the appropriate lab contact person via e-mail with the following information – name and phone number of sender (and company), tracking number and excel file containing the specimen list. It is the sender's responsibility to ensure that a person at the Viral Hepatitis lab is aware of the shipment.

Specimen Receiving:

1. Examine the address label to ensure the package is received at the correct location.
2. Examine the box for leakage or damage. If the box appears to have leaked, take the box to a biosafety cabinet or a contained area and perform the remaining functions there.
3. Open the box carefully. Look for a specimen ID list. Write today's date and your initials on the top of the specimen ID list.
4. Compare the list with the items inside the container. Be sure to check that each tube is appropriately labeled.
5. Check to see if all of the specimens are frozen and whether any tubes have cracked.
6. Place the specimens in the freezer. Refer to freezer chart for location.
7. After the specimens are put away in the freezers, write the location of the tubes on the specimen ID list next to today's date and your initials.
8. E-mail or call the sender letting them know that the specimens were received.
9. Collect all of the paperwork and staple them together. Place them in the specimen receiving binder or in the supervisor's mailbox.

There will be more detailed instructions for specimens received for each research study. However, all specimens received in the lab should follow this general format. If they do not, the supervisor will then notify the sender.

III. Specimen Shipping and Receiving – HALT-C

The following list of instructions should serve as an amendment to “Specimen Shipping and Receiving” when specimens for HALT-C specimens are sent to UW hepatitis lab.

The day specimens are shipped to UW, e-mail mchung@u.washington.edu, srchang@u.washington.edu, and rshankar@u.washington.edu with the excel file of specimen ID list and Federal Express tracking number.

When the specimens arrive in the lab, e-mail will be sent to haltc@bbii.com and halt-c@neri.org confirming the receipt of specimens and the condition of the package.

For each patient time point, there should be 3-4 aliquots of that specimen.

If the specimens do not comply with “Specimen shipping and receiving” contact BBI’s Michelle DeGrosky at 301-208-8100 ext 196. All calls will be documented in the HALT-C correspondence binder.

Place the specimens in following order. Use a 100space divider in each of the freezer boxes and label the outside of the box with the correct box number. The box numbers and specimen numbers are in sequential order with respect to date when the specimens were received. The first sets of specimens are in Box #1. When the box gets full, proceed to label the next box #2, etc.

Box map:

Spec1	Spec2	Spec3	Spec4	Spec5	Spec6	Spec7	Spec8	Spec9	Spec10
Aliq 2	Aliq 2	Aliq 2	Aliq 2	Aliq 2	Aliq 2	Aliq 2	Aliq 2	Aliq 2	Aliq 2
Aliq 3	Aliq 3	Aliq 3	Aliq 3	Aliq 3	Aliq 3	Aliq 3	Aliq 3	Aliq 3	Aliq 3
Aliq 4	Aliq 4	Aliq 4	Aliq 4	Aliq 4	Aliq 4	Aliq 4	Aliq 4	Aliq 4	Aliq 4
Spec11	Spec12	Spec13	Spec14	Spec15	Spec16	Spec17	Spec18	Spec19	Spec20
Aliq 2	Aliq 2	Aliq 2	Aliq 2	Aliq 2	Aliq 2	Aliq 2	Aliq 2	Aliq 2	Aliq 2
Aliq 3	Aliq 3	Aliq 3	Aliq 3	Aliq 3	Aliq 3	Aliq 3	Aliq 3	Aliq 3	Aliq 3
Aliq 4	Aliq 4	Aliq 4	Aliq 4	Aliq 4	Aliq 4	Aliq 4	Aliq 4	Aliq 4	Aliq 4

Put the boxes in the following order in the cane.

Write the location of the box and cane on the specimen ID list next to your signature and date.

Cane map:

"HALT-C Cane 1 (etc.)"

Box 1	6	11	16
2	7	12	17
3	8	13	18
4	9	14	19
5	10	15	20

Front of freezer

The canes for HALT-C are located in Form 3. The canes are ordered from right to left. 1st cane being furthest right and so on.

Place all paperwork in the "HALT-C Specimens Received Log" binder.

All boxes from BBI should contain an extra flap to tape over the biohazard stickers. Place this flap over the stickers and return the empty Federal Express containers back to BBI charging their Fed-ex account number: 253339314.

Federal Express forms should be filled out with following information:

1. Place your name in pre-filled out Fed-Ex form. Enter today's date and your phone number.
2. Leave the internal billing reference blank.
3. Fill in recipient's name and information – this should be the same information printed on the extra flap provided by BBI.
4. Check off FedEx Standard overnight.
5. Check off "Other pkg".
6. Check NO for dangerous goods
7. Payment: Charge to recipient with their account #253339314. Enter "1" for the package number and "2 lbs." for weight.
8. Leave number 8 blank.

Put the sender's copy in the "Specimens received log" binder and take the box to the mailroom on 1st floor, HMC 1EC.

IV. Prioritization and Testing Schedule of HALT-C Specimens

The following time points in the listed order have highest priority for HCV qualitative testing by COBAS Roche Amplicor, v.2.0. When the following specimens are received in lab, qualitative result should be available within 72 hours.

1. Week 20 (duplicate sample) (seq. 101-104)
2. Confirmatory specimens from W20 Responders who have tested positive (duplicate sample) (seq. 150-153)
3. All W20 Responders (seq. 105-108)
4. Screening specimens from Express group (duplicate sample) (seq. 101-104)

The following time points have highest priority for HCV quantitative test by COBAS Amplicor HCV Monitor, v.2.0. When the following specimens are received in lab, quantitative test should be performed in the next available run in the listed order.

1. Screening specimens from Lead-in Group (seq. 101-104)
2. Week 12 (seq. 105-108)
3. W20 Responders with positive HCV qualitative result (seq. 105-108)

All other specimens will be tested in the order they are received and according to availability of space.

Testing Schedule

HALT-C specimens arrive at Harborview Medical Center on Tuesdays and Thursdays from the BBI Repository. Each specimen is labeled with a unique BBI identification number and aliquot number. All results for HALT-C HCV-RNA specimens will be data entered onto Form 31 except for the confirmatory test (seq. 150-153) which will be data entered onto Form 37. Results from the genotype test will be data entered onto Form 33. If a quantitative test is performed on specimens with a qualitative result already entered into the DMS, Form 32 will be expected for the quantitative result, except for confirmatory specimens (seq. 150-153).

Lead-in Group

S00 Aliquot #s: 101-104

These screening specimens are drawn prior to enrollment into HALT-C clinical trial. They are to be tested using the HCV quantitative assay after they have been diluted with negative serum at 1:100. Report the result x 100 to NERI. If the result is “result lo”, a new aliquot should then be tested, undiluted. If a numerical result is available, report this result to NERI. If the result is again “result lo”, test using Amplicor HCV qualitative assay. Report the result to NERI as negative or positive, according to qualitative test result.

W00 Aliquot #s: 105-108

These specimens are the patients’ baseline time point prior to receiving any treatment. They are to be tested using the HCV quantitative assay after it has been diluted with negative serum at 1:100. If this specimen is quantifiable after it has been diluted, report the result out x 100 to NERI. If the result is “result lo”, test a new aliquot, undiluted. If a numerical result is available, report the result to NERI. If not, then test a new aliquot by Amplicor HCV qualitative assay. Report the result to NERI as negative or positive, according to qualitative test result.

W02 Aliquot #: 109

These specimens are for HCV genotype. Only one aliquot is provided. Since the InnoLipa kits have not been validated, these specimens shall be stored until testing can be performed at a later time.

W12 Aliquot #s: 105-108

These specimens are drawn during the “Lead-in Phase” and are a prelude to W20 specimens. Quantitation of these specimens is crucial in following the patients’ progress during this phase. These specimens are to be tested using the HCV quantitative assay after it has been diluted with negative serum at 1:100. If this specimen is quantifiable after it has been diluted, report the result x 100 to NERI. If the result is “result lo”, test a new aliquot, undiluted. If a numerical result is available, report the result to NERI. If not, then test a new aliquot by the Amplicor HCV qualitative assay. Report the result to NERI as negative or positive, according to qualitative test result.

W20 Aliquot #s: 101-104

The W20 result determines whether or not the patient enters the “Randomized” Phase of the study or the “W20 Responder” Phase. Thus, the turn around time for these specimens is crucial. When BBI notifies the lab that W20 specimens are on their way, a qualitative worksheet is generated to include all incoming W20 specimens. Within 24 hours of the specimen receipt, qualitative testing in duplicate is performed on these specimens. If the duplicate testing results are concordant, report the result to NERI immediately. If the results are discordant, refer to W20 algorithm. Other testing may need to be delayed, as these are the highest priority specimens.

W24 Aliquot #s: 105-108

These specimens will be tested around the time of a patient’s M12 or W48 study visit. Specimens will be sent to the Virology Lab from BBI following shipment of these later time point specimens.

Forms for the Lead-In Group

All results will be entered onto Forms 31, 32 and 33.

W20 Responder Group

W36, W48, W60 and W72 Aliquot #s: 105-108

These specimens will be drawn from patients who responded to Lead-in Phase treatment and have tested negative by HCV qualitative test at week 20. These samples should be tested qualitatively using the Amplicor HCV assay first. If the result is positive, then quantitative testing should be performed. Test diluted at 1:100 first, then undiluted. If any of these specimens are positive, expect a confirmatory specimen from the clinical sites. See Confirmed Specimens from W20 Responders who have tested positive. The qualitative result will be recorded on Form 31 and if quantitative test is performed, this result will be recorded on Form 32.

Confirmatory Specimens from Breakthrough/Relapser Group

W36^{Repeat}, W48^{Repeat}, W60^{Repeat} and W72^{Repeat} Aliquot #s: 150-153

These specimens are sent to our lab to confirm any positive specimens from the W20 Responder Phase of the trial. These specimens should be tested qualitatively in duplicate by COBAS Amplicor HCV v.2.0 assay first. The results from the test will be sent to NERI when they are available. The receipt of these specimens will be dependent on when the previous result was released and when the patient is scheduled to come in next. The results will be recorded on Form 37.

Forms for the Breakthrough/Relapser Group

After an initial positive result at W36, W48, W60 or W72, the patient has the option of scheduling a confirmatory HCV RNA blood draw at or before the next scheduled study visit. If the patient chooses to have the confirmatory blood draw at the next scheduled visit, the standard aliquot Form 74 for W20 Responders and Form 31-HCV RNA Form will be used. Patients found to be eligible for the trial will be brought in for a randomization (R00) visit. They will then come in for M09-M54 visits. If the patient chooses to schedule a blood draw prior to the next study visit, Form 76-Repeat HCV RNA Aliquot Form will be used and results will be reported on Form 37-Repeat HCV RNA Results. These confirmatory specimens will have sequence numbers 150-153.

Randomized Group

M12, M18, M24, M30, M36, M42, M48 and M54 Aliquot #s: 105-108

These specimens will be drawn from patients who have tested positive by HCV qualitative test at week 20 or have been randomized to the main study. This phase also includes Express patients. These specimens should be monitored quantitatively by testing the diluted specimen at 1:100 and then testing undiluted if the result is below the detection limit with dilution. If the result remains "result lo" after it has been tested undiluted, test a new aliquot by Amplicor HCV qualitative assay.

Express Group

These specimens will be drawn at Screening from patients who have tested positive by HCV qualitative test after at least 24 weeks of interferon treatment outside of the Lead-in Phase. These specimens should be monitored quantitatively by testing the diluted specimen at 1:100 and then testing undiluted if the result is below the detection limit with dilution. If the result remains "result lo" after it has been tested undiluted, test a new aliquot by Amplicor HCV qualitative assay.

Forms for the Express Group

Samples generated from the Express Patient group at the Randomization Visit (R00) are similar to those generated at the W00 and W24 visits. Form 77-Randomization Visit Aliquot Form will be

used and results will be reported on Forms 31 and 33. An e-mail will be sent to the virology lab from the DMS when an express patient comes in for the Screen visit 1.

V. Monthly Testing of HALT-C Specimens by Roche Amplicor Qualitative Test for Qualitative Control, v.1.0

Purpose:

The primary function of the central virology laboratory is to provide accurate and timely information from patient specimens. Performing monthly qualitative tests on randomly selected patient samples can prevent errors and help assess the validity of runs and kits.

Procedure:

Nine random specimens tested in the previous month by the Roche Amplicor Qualitative or Roche Amplicor Monitor Assays for HCV RNA will be re-tested by HCV Roche Amplicor qualitative test. Among the nine specimens will be at least two negative specimens, at least one in the quantitative range of 600 to 500,000 IU/ml and at least one with a titer above 500,000 IU/ml. The remaining 5 specimens will be selected at random for re-testing. This run will be set up at end of each month and each technologist will rotate to perform the tests.

VI. Duplicate HCV Testing Algorithm for HALT-C, v.2.0

Screening specimens from “Express patients”, confirmatory specimens from positive responder group and all week 20 specimens will be extracted and tested in duplicate by Roche Amplicor Qualitative test v.2.0. In order for a W20 result to be reported to the DCC as positive, either a positive HCPCR result must be obtained from two different vials in two separate runs or a positive HCPCR result and a viral titer must be obtained from two different vials. Refer to algorithm for details (following). All results must be verified with another qualified technologist before reporting. Negative results must have had a positive internal control (IC).

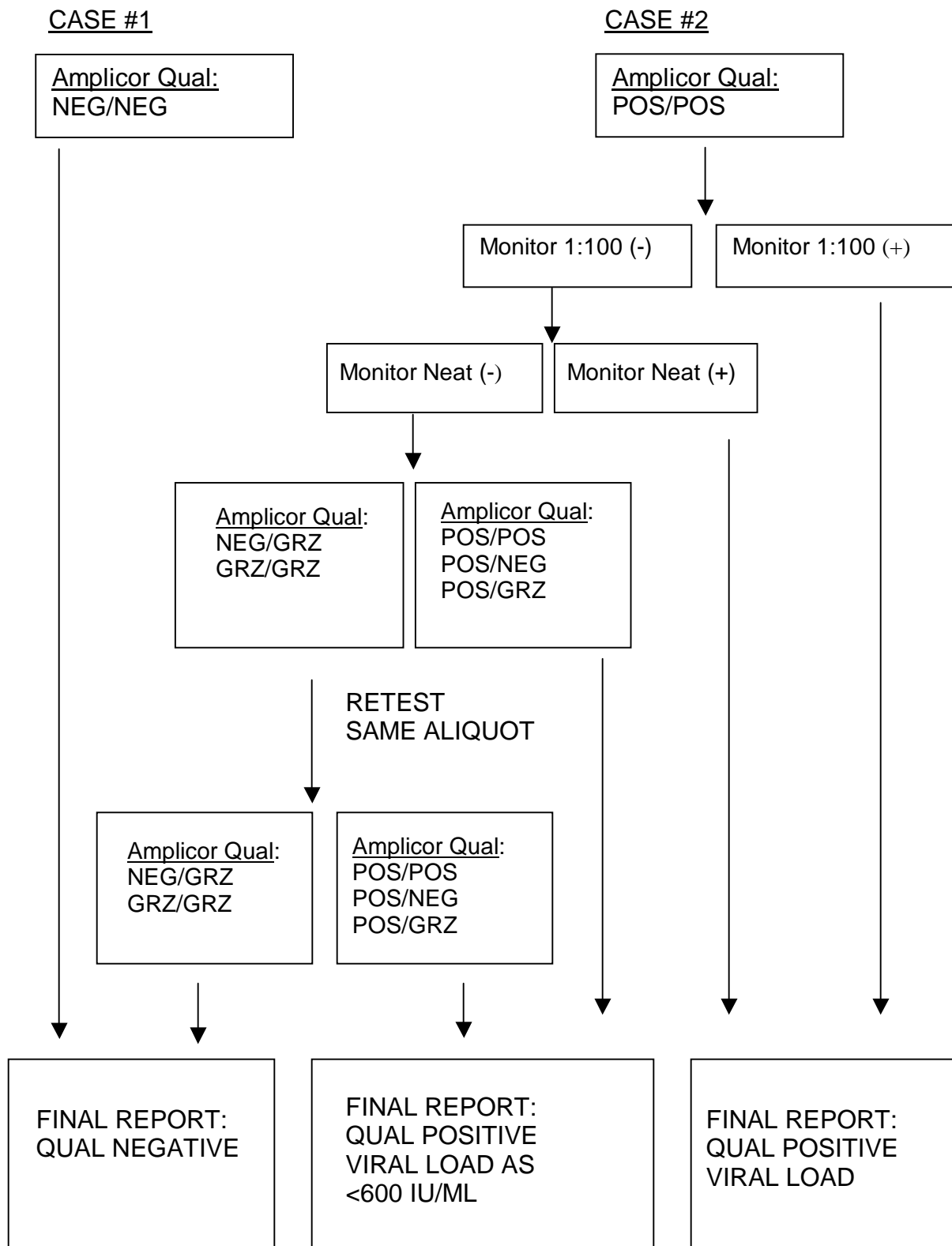
If duplicates from the first aliquot indicate the presence of inhibitors, test a second aliquot in duplicate. If one or both of these duplicate results from the second aliquot contain inhibitors, contact BBI for a new specimen. In the meantime, specimens with inhibitors present will be tested by an in-house method of qualitative testing for hepatitis C virus. This method is comprised of three distinct PCR assays using oligonucleotide primer sets from three separate regions within the viral genome: 1) highly conserved 5'UTR, 2) relatively conserved Core and 3) heterogeneous Envelope 1 (E1) region. If any of these tests is positive for HCV RNA, the sample will preliminarily be considered positive. The new specimen will be tested in duplicate after it is received, and will serve as a confirmatory result.

NEG = Negative Result

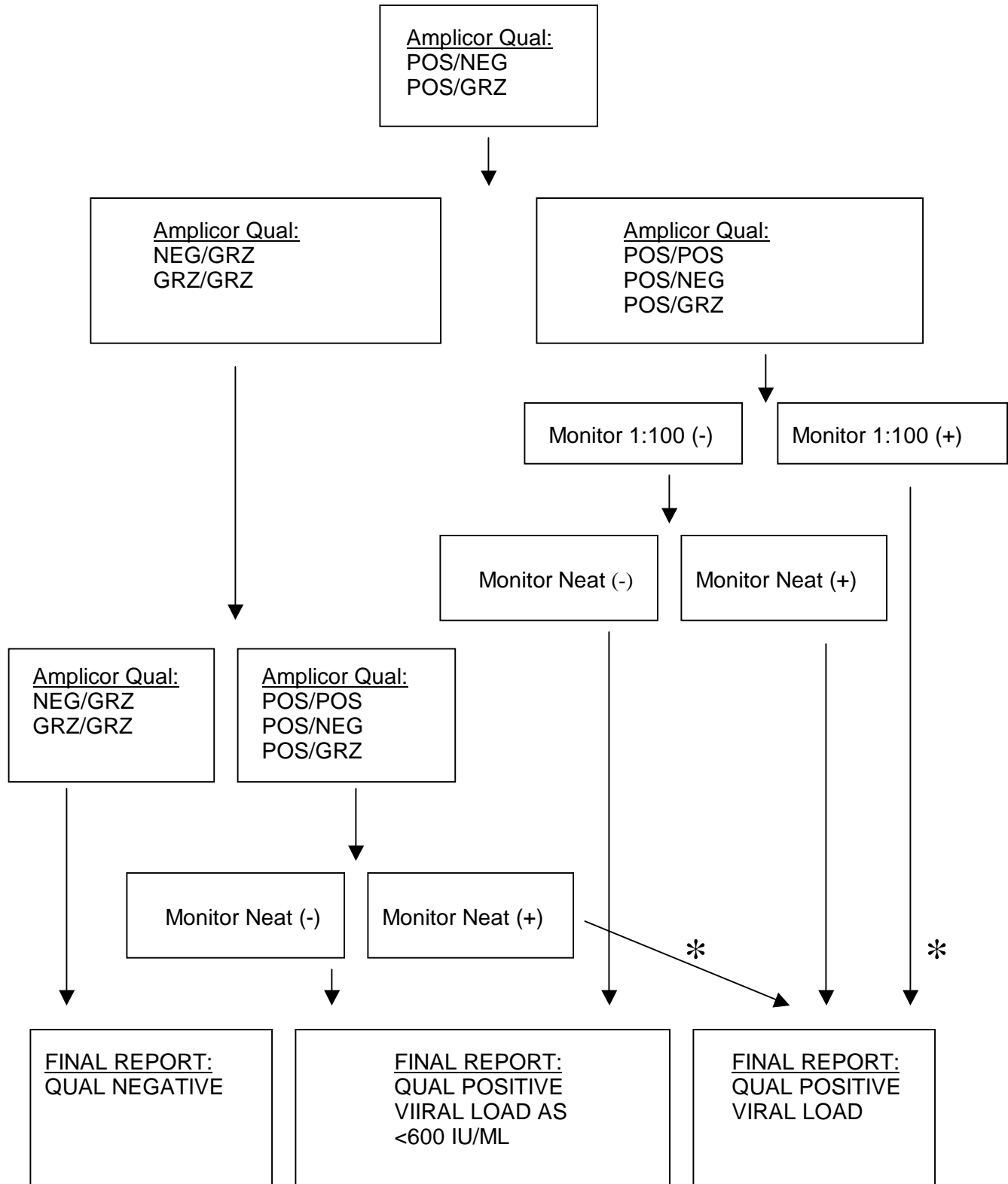
POS = Positive Result

GRZ = Grey Zone Result

Duplicate HCV Testing Algorithm for HALT-C, v.2.0

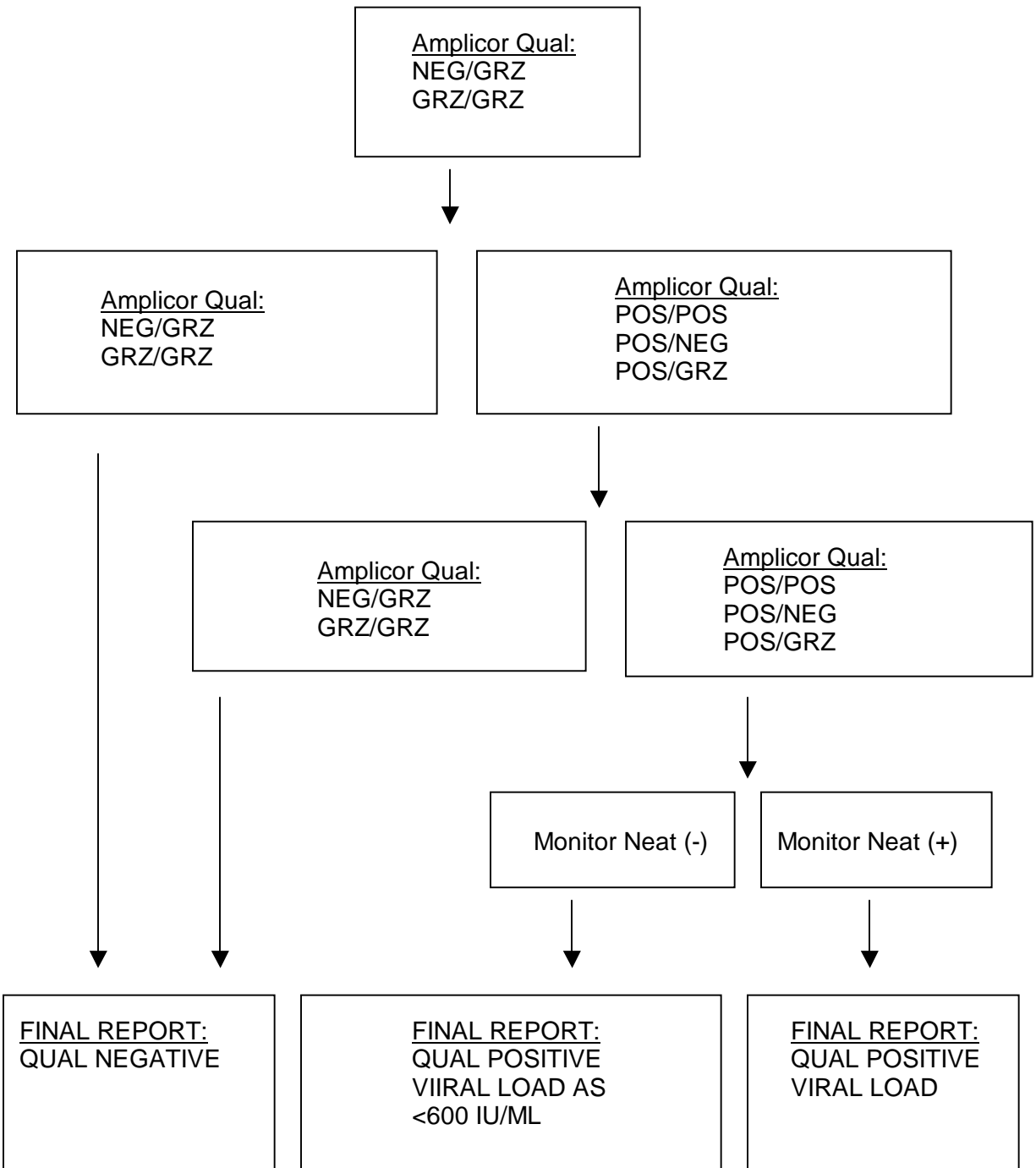


CASE #3



* may require further investigation

CASE #4



VII. Qualitative Detection of Hepatitis C Viral RNA by Polymerase Chain Reaction by COBAS AMPLICOR HCV 2.0 Test, v.2.1

Principle:

The COBAS Amplicor HCV test, v.2.0 is the first PCR-based test to detect Hepatitis C Virus that has been approved by the U.S. Food and Drug Administration. This test is an in vitro test for the detection of Hepatitis C virus in human samples on the COBAS AMPLICOR analyzer. The test utilizes reverse transcription of target RNA to generate complementary DNA, nucleic acid amplification of target cDNA by the PCR and nucleic acid hybridization for the detection of HCV RNA in human serum and plasma.

The COBAS AMPLICOR HCV Test, v.2.0 is based on five major processes: specimen preparation, reverse transcription of target RNA to generate into cDNA, polymerase chain reaction (PCR), hybridization of the amplified product to a specific nucleic acid probe, and detection of the amplified product by color formation.

For a more detailed description of this test, please consult the package insert

Turn around time:

The test will be performed as each batch (9 or 20 specimens) is filled or as results are needed. The maximum number for each batch is 22.

Specimens:

Serum from whole blood separated within 2-4 hours of collection. (See specimen criteria/collection)

Equipment/Materials needed:

- HCV qualitative worksheet
- COBAS Amplicor HCV Qualitative A-ring map
- COBAS AMPLICOR HCV test, v.2.0 – Specimen Preparation Kit v.2.0
 - Controls Kit, v.2.0
 - Amplification Kit, v.2.0
 - HCV Detection Kit, v.2.0
 - Detection Reagents Kit
 - Conjugate Detection Reagent
 - Wash buffer
- COBAS AMPLICOR A-ring fitted with 12 A-tubes
- COBAS AMPLICOR A-ring holder
- Plastic resealable bag
- Eppendorf Repeater pipette with 1.25mL Combitip (sterile, individually wrapped)
- vortex
- 1.5 ml sterile screw cap tubes (Sarstedt)
- Tube racks
- 50 ml Falcon tubes
- Heat blocks, adjustable from ambient to 100°C
- Microcentrifuge with maximum rcf of 14,000 x g
- Precision pipettors (adjustable to deliver 1-20 µl, 20-200 µl, and 200-1000 µl)
- Sterile transfer pipettes
- COBAS Amplicor Analyzer
- PC with Amplilink software
- Plastic resealable bag
- Dry heat block
- Sterile 5mL serologic pipettes

Pipetaid

Reagents:

All reagents contained within the kits are stable unopened at 2-8°C until their expiration date. Once the cassettes are opened, please label with a red pen the date each cassette was opened.

1. Isopropyl alcohol (made in clean room)

Filter sterilize before use. Store at room temperature up to 6 months.

2. 70% Ethyl alcohol

Use fresh 70% ethanol for each assay - pour 35 mls ethanol (absolute) into a 50 ml conical tube, then add 15 mls of DEPC water. Mix by inverting before use. Store up to 24 hours.

3. DEPC treated water (made in reagent room)

Using a fume hood, add 100 µl diethyl pyrocarbonate (DEPC) per 100 ml deionized water. Mix for 1 hour at room temperature and filter through 0.2 µm filter. Store at 4°C for up to 6 months.

4. HCV Probe Suspension (made in product room)

Vortex CX PS1 until it is fully resuspended. HCV probe suspension is made by adding 2.5 ml of CX PS1 to a cassette labeled CX4. Do not pipette up and down. Once they are mixed, the working reagent is stable for 30 days at 2-8°C or maximum of 6 instrument cycles (12 hours per cycle). Put the date made on the side of the cassette with a red pen.

5. Internal Control Probe Suspension (made in product room)

Vortex IC PS1 until it is fully resuspended. Internal control probe suspension is made by adding 2.5 ml of IC4 PS1 to a cassette labeled IC4. Do not pipette up and down. Once they are mixed, the working reagent is stable for 30 days at 2-8°C or maximum of 6 instrument cycles (12 hours per cycle). Put the date made on the side of the cassette with a red pen.

6. Substrate (made in product room)

Working substrate must be prepared each day by mixing 5mL of SB3 to cassette labeled SB. They are light sensitive reagents.

Controls:

One positive, one sensitivity, and one negative control are required for a run. If running more than one ring at a time, a second negative control should be added.

1. HCV negative control, HCV (-) C, is a kit negative control. This control is required in all runs.

2. In house HCV negative control is plasma from a HCV negative donor. It has previously been tested negative by this assay. This control is required if running more than 1 ring.

3. HCV positive Control, HCV (+) C, is a kit positive synthetic RNA. One positive control is required for each run.

4. Negative Human Plasma is used for diluting the controls.

5. HCV sensitivity Control is an in-house diluted positive specimen. It is the last dilution which

most of the technologists will get a positive result when tested by COBAS Amplicor v.2.0.
Treat this control as specimen throughout the procedure.

Procedure:

In each of the rooms, there is a 1-page procedure (see figure 2) taped to the wall near the work station. Please refer to this when performing the assay. For a more detailed procedure, please refer to the package insert.

There are dedicated rooms and equipment for each of the procedures. Move unidirectional only from area 1 (clean room) to area 2 (product room) when performing this assay. When aliquoting specimens, only open one tube at a time. Do not return unused specimen/reagent to the original container. Use aerosol resistant (plugged tips) when indicated to help prevent carry-over contamination.

Before proceeding any further, make sure to have your worksheet and the COBAS Amplicor HCV Qualitative ring map (figure 1) ready.

A. Reagent Preparation (PCR Hood)

1. Clean the PCR hood and pipettor to be used with 70% ethanol. Turn on the UV light source for at least 15 minutes prior to use to decontaminate the area
2. Looking at your worksheet, determine the appropriate number of A-rings needed for patient specimens and controls.
3. Prepare enough working master mix by adding 100 µl of HCV Manganese Solution to one tube of HCV Master Mix. Recap the Master Mix tube and mix well by inverting the tube 10-15 times. The pink dye in the Manganese Solution is used for visual confirmation that the Manganese Solution has been added to the Master Mix. Discard remaining Manganese Solution.
4. Place the A-ring(s) in the A-ring holder(s).
5. Pipette 50 µl of working master mix to each A-tube using a repeater pipette. Discard any remaining working master mix.
6. Place the open A-ring(s) containing working master mix back in its resealable plastic bag. Seal securely and store at 2 to 8°C until use. The working master mix is stable for 4 hours at 2-8°C in A-tubes sealed in the plastic bag.

B. Sample Preparation (Clean Room Biological Safety Cabinet)

1. Turn on heat block to 60°C.
2. Label one 1.5ml Sarstedt tube for each patient sample and 2 additional tubes for kit's positive and negative controls. The sensitivity control and in-house negative control should be treated as patient samples.
3. Warm lysis reagent to 25 - 37°C and mix thoroughly to redissolve precipitate.
4. Prepare working lysis reagent by adding 100 µl of well vortexed HCV IC, v.2.0 to the Lysis reagent. Keep this reagent separate from the lysis reagent not containing the HCV IC. Mix well. Discard remaining IC. The working Lysis Reagent is stable for 8 hours at room temperature.
5. Thaw serum or plasma specimens at room temperature and vortex for 3 to 5 seconds.
6. For each specimen or control, using a repeater, dispense 400ul working Lysis buffer into a 1.5 ml Sarstedt tube.
7. Prepare the positive and negative controls as follows, the sensitivity control should be treated as patient specimen:
 - a. Vortex NHP, HCV negative and positive controls for 5-10 seconds.
 - b. Add 200ul NHP to each of the control tubes. Cap tubes and vortex 3-5 seconds.

- c. Add 20ul of each control to its corresponding tube and vortex 3-5 seconds.
8. Using a new plugged tip for each specimen, add 200ul of serum specimen or sensitivity control to each tube. Only open one tube at a time to avoid carry-over. Cap tubes and vortex well (3 - 5 seconds). Immediately place the original specimen tube in the freezer or the refrigerator.
9. Incubate tubes containing serum and working lysis reagent for 10 minutes at 60°C heat block. Make sure to note the temperature on the worksheet.
10. Using a repeater, carefully add 600ul isopropanol to each tube. Only open one tube at a time. Vortex well (3 to 5 seconds). Incubate at room temperature for 2 minutes.
11. Put an orientation mark on each tube and place into the microcentrifuge with the orientation mark facing outward, so that the pellet will align with the orientation mark. Centrifuge at maximum speed in the microcentrifuge for 15 minutes. During centrifugation, make 70% ethanol by adding 35ml of ethanol to 15ml of DEPC water in a 50ml falcon tube.
12. Using a new transfer pipette for each specimen, carefully aspirate the supernatant without disturbing the pellet (which may not be visible). Be careful to remove as much supernatant as possible without disturbing the pellet. Discard the supernatant in a container containing bleach.
13. Opening one tube at a time, add 1 ml 70% ethanol using a repeater, re-cap, and vortex well (3 to 5 seconds).
14. Place the tubes in the microcentrifuge so that the pellet will align with the orientation mark. Centrifuge at maximum speed for 5 minutes.
15. Using a new transfer pipette for each specimen, carefully aspirate the supernatant without disturbing the pellet (which should be clearly visible). Be careful to remove as much supernatant as possible without disturbing the pellet.
16. Recap the tubes and spin at maximum speed for 3-5 seconds, making sure to align the orientation marks.
17. Using a nonplugged pipette tip, remove all the residual ethanol from each tube.
18. Add 200uL of specimen diluent to each tube. Break apart the pellet as much as possible using a P200 pipettor fitted with a plugged tip. Vortex for 10 seconds. Analyze the processed specimens within 3 hours or freeze the processed specimens at -70oC for up to one month, with no more than two freeze-thaws.

If storing the RNA for later amplification and detection, make note on the worksheet regarding which -70°C freezer the RNA is stored in.
19. Make sure to record the run number, date and your initials on the first tube of RNA for reference.
20. Add 50 µl each processed specimen to the appropriate A-tube containing 50 µl master mix using a plugged tip. Be careful to avoid transferring any precipitated material that may not have been resuspended. Cap the A-tubes. **Amplification must be started within 45 minutes** of the time that the processed specimens and controls are added to the A-tubes containing master mix.

C. Reverse Transcription, Amplification, and Detection (PCR Product Room)

1. Maintenance of COBAS analyzer:

- a. Turn on the COBAS analyzer and then activate Amplilink.
- b. When prompted for user name, type in your initials.
- c. Perform maintenance. Click on the service icon on Amplilink. Then click on the Services Actions tab. Examine each task and determine which service actions are due. Highlight the tasks that are due on the list and as you perform it, click on "update".

For daily service, refer to the due date column for the next date to perform the daily actions.

For weekly and 2-month service, the background of the service icon will turn yellow and eventually red. Maintenance should be performed while the icon is still yellow. Do not proceed to running any tests when this icon is red.

After maintenance is complete, click on "update" to display the update service action entry dialogue box. The user may enter free text in the note box.

- d. When system prime is required, click on "tools", then "prime". Click either normal or extend to begin priming. If more buffer is made, place the empty buffer bottle next to the reservoir so the next person will know the lot number of the wash buffer. Also write initial and date on the tape on the carboy.
- e. When Analyzer's syringes require changing, click on "tools", then "syringe exchange" to position the syringe plunger to the up position. Replace the syringe tips as outlined in the operator's manual and click on "yes" when asked if the syringe tips are being replaced.
- f. Click on OK to record the servicing in the Service Log and to reset the counter in the Service Action folder. Make sure all the scheduled maintenance is done before leaving the service menu. Transfer this information to the paper copy located in the blue binder labeled "COBAS Maintenance Log".

2. Instrument Loading and System Operation

For each A-ring used for qualitative HCV testing, the following reagents (minimum number of tests per ring) are required.

D-cups	(26)
Denaturing Solution (DN)	(12)
Conjugate (CN4)	(24)
Substrate (SB)	(26)
HCV Probe Suspension (CX4)	(12)
Internal Control Probe Suspension (IX4)	(12)

- a. Examine the quantities of reagents on board the COBAS AMPLICOR analyzer as well as any open cassettes in the refrigerator. To determine how many tests are available in each of the cassettes, on Amplilink click on the "analyzer" icon and click on the "cassettes" tab. Double click on the abbreviated reagent to display the expanded list. Click on the cassette barcode of interest and the "cassette status" will appear with expiration date, # of tests available, and when it was first placed on the COBAS analyzer.
- b. Prepare enough reagents to complete a work list. Please refer to "reagents" above for directions for making reagents.
- c. Place each cassette in its appropriate rack. DN4, CN4 and working substrate should go on the generic reagent rack. IX4 and CX4 belong on the specific reagent rack. If placing a new cassette on the rack, record today's date on the cassette with

- a red pen.
- d. Identify the reagent racks as generic or test specific using the keypad or barcode scanner as described in the Operator's Manual for the COBAS AMPLICOR analyzer.
- e. Configure the reagent racks by inputting reagent positions and lot numbers into the instrument using the keypad or barcode scanner as described in the Operator's Manual for the COBAS AMPLICOR analyzer. Do not print this list.
- f. Load the reagent rack onto the instrument using the keypad or barcode scanner as described in the Operator's Manual for the COBAS AMPLICOR analyzer. Skip the printing instructions. Make sure that each reagent cassette is in its assigned position and that each cassette fits tightly into its rack.
- g. As the information is saved on the analyzer, the racks and the cassettes should be displayed on the Amplilink screen. Be sure to take note the cassette colors. If the cassette is green, it indicates that the cassette is loaded and contains greater than 10 test counts. If the cassette is yellow, it indicates that the cassette is loaded and has expired or contains volume sufficient for 10 or less tests. Make sure to add an extra cassette to replace the insufficient cassette. If the cassette is red, there are 0 counts remaining or there is a volume mismatch. Discard any red cassettes.
- h. Place the D-cup rack on the D-cup platform. Two D-cups are required for each specimen and control and two D-cups are required for each Working Substrate to allow for blanking by the COBAS AMPLICOR analyzer.
- i. Place the A-ring(s) into the thermal cycler segment(s) of the COBAS AMPLICOR analyzer.
- j. Load the A-ring(s) into the Analyzer using the keypad or barcode scanner as described in the Operator's Manual for the COBAS AMPLICOR.

Once the A-rings are loaded into the Analyzer, click on the "analyzer" in Amplilink and the loaded A-rings should be displayed on screen.

- k. Create an A-ring work list by clicking on the "order" icon in Amplilink. First, click on the "+" button on top of the screen. This empties the summary list and lets you enter your own. Enter the A-ring ID where it prompts you to. Click on the profile desired under "Profiles and Tests". **MAKE SURE THE PROFILE MATCHES THE TESTS.** Enter the sample ID. When completed, **double check your work.** Then click "save". For each specimen, make sure to order 2 tests – HCX and HXC.
- l. Make sure the COBAS analyzer is closed.
- m. Press "start" in the status menu. It will then ask if you would like to be in parallel mode or basic mode. Note: parallel mode stops after the rings in TCA and TCB have completed the denaturation and DP1 and DP2 have completed the detection. Basic mode performs amplification and detection without stopping after denaturation. Choose either parallel or basic, then press okay. The analyzer will then perform a load check to determine if it has all the reagents required for performing the tests ordered. If the load check passes, print the load check by clicking on the printer icon. Make sure the printed load check stays together with the worksheet and the A-ring map.

D. Data Review

1. After the run is complete, click on the "results" icon on Amplilink. Then select the appropriate A-ring number. The results for that ring will be displayed on screen. It will ask if you would like to accept all or some of the results. Accept all results and print a copy of the results by clicking on the "printer" icon. Attach the results to rest of your

forms. The result form contains the reading at A660 for the blank and the sample. Underneath, it will give an interpretation of "POS", "NEG" or "GZN".

2. Check the entire printout carefully for flags and comments to ensure that the run is valid.

3. For the run to be valid, all of the controls must be within the following range:

All negative controls are < 0.1 at 660nm

Both positive and sensitivity controls are ≥ 1.0 at 660nm.

The sensitivity control has to be positive.

4. If the HCV (-) control is greater than or equal to 0.1, the entire run is invalid. Repeat the entire process including sample prep, amplification and detection. If this occurs again, contact Roche Technical service at 1-800-428-2336.
5. If the HCV (+) control is less than 1.0, the entire run is invalid. Repeat the entire process including sample prep, amplification and detection. If this occurs again, contact Roche Technical service at 1-800-428-2336.

For sample results, refer to the following chart:

<u>HCV Sample Result</u>		<u>IC Sample Result</u>		<u>Interpretation</u>
A ₆₆₀	Cobas Flag	A ₆₆₀	Cobas Flag	
< 0.15	Negative	≥ 0.15	Positive	Specimen is negative
< 0.15	Negative	< 0.15	Negative	Specimen is Inhibited
≥ 1.0	Positive	ANY	ANY	Specimen is positive
$\geq 0.15, < 1.0$	GZ 0.15-1.0	ANY	ANY	Equivocal

6. If a specimen is **inhibited**, process the second aliquot of sample and repeat the test.
7. If the result is **equivocal**, repeat the test in duplicate using the second aliquot of the patient sample. If both repeat test results are ≥ 0.15 A660 that specimen should be considered positive for HCV RNA. Samples with one or both replicate test results < 0.15 A660 should be considered a presumptive negative for HCV RNA provided that the IC results for both replicates are ≥ 0.15 for A660.

E. Results

1. Only report the positive and negative results.
2. If the specimen is **inhibited** again, process another aliquot in duplicate and repeat the test.
3. If a specimen was repeated in duplicate, both repeat test results need to be ≥ 0.15 A660, for that specimen to be considered positive for HCV RNA. Samples with one or both replicate test results < 0.15 A660 should be considered a presumptive negative for HCV RNA provided that the IC results for both replicates are > 0.15 for A660.
4. If specimen was tested in duplicate, see duplicate testing algorithm for interpretation of results.

F. Sensitivity

The sensitivity of the COBAS AMPLICOR HCV TEST v2.0 has been determined by the manufacturer to be 100 IU/ml for serum.

Reference:

Refer to Roche COBAS Amplicor HCV v2.0 package insert.

Archiving Results

All accepted results should be archived. The specimens tested for HALT-C should be archived by ring ID under the worksheet title into a folder labeled "HALT-C *date*". Others will be archived under their appropriate folders. The entire folder under HALT-C will then be copied to a disk and sent to NERI.

Miscellaneous

1. Once the results have been reviewed and validated by another technologist, the extracted RNA can be discarded.
2. Attached to each worksheet should be the A-ring map, load check from COBAS analyzer, and the results. All forms should be completed and initialed by the tech running the assay prior to review by the second technologist.
3. Make sure to clean up after yourself and restock any empty cabinets.

Figures

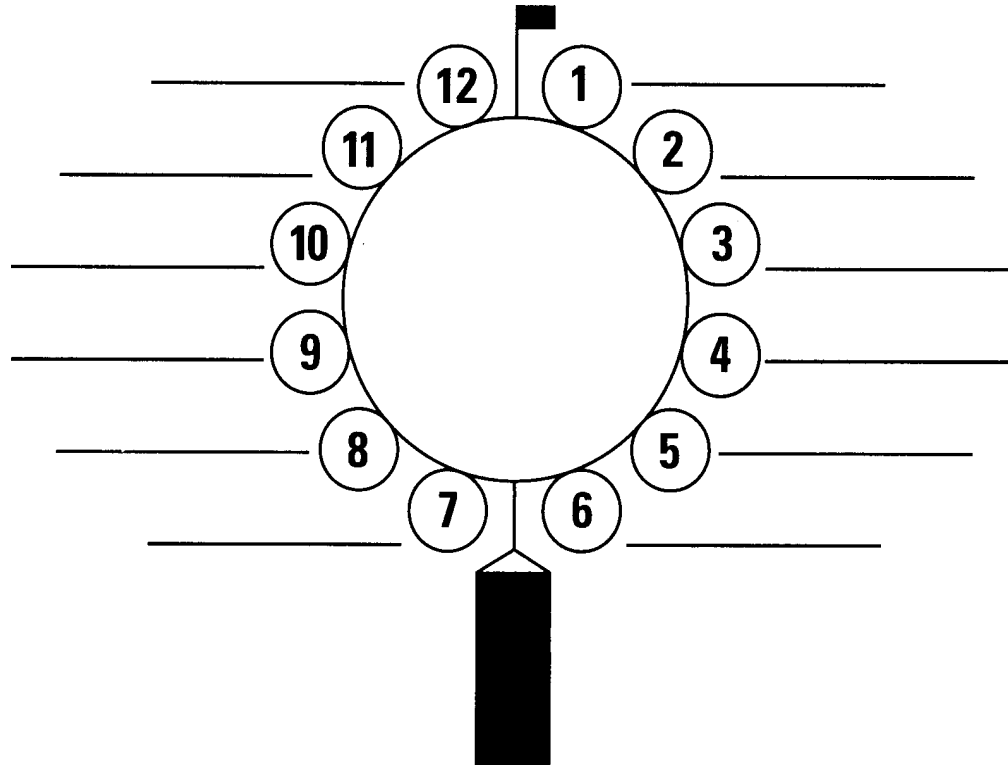
Figure 1: COBAS AMPLICOR™ HCV Qualitative A-RING MAP

Figure 2: COBAS AMPLICOR™ HCV QUALITATIVE TEST - VERSION 2.0

COBAS AMPLICOR™ HCV Qualitative A-RING MAP

Ext. Date: _____ Amp. & Detect: _____

OPERATOR: _____
Run #/Worksheet #: _____
A-RING ID # : _____



Assay	Lot #	Exp. Date
Amplification Kit		
Specimen Preparation Kit		
HCV (CX) Detection P/N:1111140		
Internal Control (IC) kit #0757608		
Detection Reagent Kit P/N 20757470		
Conjugate Detection P/N 20764213		
Wash Buffer P/N 20759899		
Controls Kit v.2.0		

*Check to make sure specimen freezer temp, clean room refrigerator, and product room refrigerator temperatures are marked off **Yes** **No**

LOAD CHECK: Pass ☐ Fail ☐

If load check fails, STOP Transfer, correct problem, restart.

Instrument QC OK (no error messages) ☐

List any errors here: _____

COMMENTS:

COBAS AMPLICOR™ HCV QUALITATIVE TEST - VERSION 2.0
For Research Use Only - Not For Use In Diagnostic Procedures

Cobas Amplicor™ PCR Reagent Preparation - AREA 1	Cobas Amplicor™ PCR Specimen Preparation AREA 2
<p>Always wear new gloves and lab coat when proceeding to next area. Amplification and detection kit must be at room temperature (RT).</p> <p>A. REAGENT PREPARATION</p> <ol style="list-style-type: none"> Determine the number of samples and controls to be tested. Vortex HCV Mn²⁺, v2.0 prior to HCV MMX, v2.0 preparation. Prepare the Working Master Mix by adding 100ul of HCV Mn²⁺, v2.0 to one vial of HCV MMX, v2.0. Mix by inverting 10-15 times. Add 50ul of Working Master Mix into each A-tube using a repeat pipettor. Do not cap the tubes at this time. Visually inspect A-tubes to ensure that the Working Master Mix has been added. Place the A-ring(s) in a resealable plastic bag and place the sealed bag in the refrigerator at 2-8°C in AREA 2 until ready to add samples. Stable for four hours. <p><u>NOTE:</u> All VORTEXING SHOULD BE DONE FOR AT LEAST 3 SECONDS UNLESS OTHERWISE NOTED.</p>	<p>A. REAGENT PREPARATION</p> <ol style="list-style-type: none"> Prepare 70% ethanol by mixing 11.0 ml of 95% ethanol and 4.0 ml of deionized water. Make fresh daily. Visually inspect HCV Lysis Reagent (HCV LYS) to make sure that it is homogeneous and free of any precipitate. Warm between gloved hands and vortex. Vortex HCV IC, v2.0 for 5-10 seconds. Prepare Working Lysis Reagent. Add 100ul of HCV IC, v2.0 to one bottle of HCV LYS v2.0 and mix well. Note: Working Lysis Reagent is stable for 8 hours at RT. Note: HCV IC, v2.0 must be added to HCV LYS v2.0 even if the HCV internal control will not be detected. <p>B. SPECIMEN PREPARATION</p> <ol style="list-style-type: none"> Complete the A-ring Map for sample and control identifications. Determine the correct number of Sarstedt 1.5 ml tubes needed for samples and controls. Label and place an orientation mark on each tube. Add 400 µl of Working Lysis Reagent to each of the labeled tubes and cap the tubes. Vortex the negative human plasma (NHP), HCV (-)C, HCV (+)C, all v2.0 for 5-10 seconds. Add 200ul of NHP to each of the two control tubes already containing 400ul of Working Lysis Reagent. Cap the tubes and vortex. Add 200ul of patient serum or plasma to the appropriate tubes containing 400ul of Working Lysis Reagent and mix well by vortexing immediately. Add 20ul of HCV (-)C and (+) C, v2.0 to each of the respective tubes containing 400ul Working Lysis Reagent and NHP and mix well by vortexing immediately. Incubate all tubes in a dry heat block for 10 minutes at 60°C ± 2°C. Mix well by vortexing. Add 600ul of 100% isopropanol to each tube and vortex immediately. Incubate all tubes for 2 minutes at RT. Position the tubes in the centrifuge so that the orientation marks are facing outward. Centrifuge tubes for 15 minutes at maximum speed (12,500 – 16,000 x g) at RT. Using a fresh fine tip transfer pipette for each sample, carefully aspirate and discard the supernatant without disturbing the pellet (pellet may not be visible). <i>Perform only one aspiration!!</i> Add 1 ml of 70% ethanol to each tube and vortex. Centrifuge for 5 minutes at RT, 12,500 – 16,000 x g. Using a fresh fine tip transfer pipette for each sample, carefully aspirate and discard the supernatant without disturbing the pellet (pellet should be clearly visible at this step). Recap the tubes and spin at maximum speed for 3-5 seconds. Remove the supernatant using a 200ul capacity pipettor with a new tip for each sample. <i>Two aspirations required!!</i> Resuspend pellet in 200ul HCV diluent (DIL) v2.0 to each tube. Vigorously scrape/break pellet apart with a P200 pipettor fitted with a P200 plugged tip (new tip for each specimen). Vortex for 10 seconds. Start amplification within 3 hours of specimen and control preparation or store frozen at -70°C or colder for one month with no more than two freeze-thaws. Remove A-ring(s) from refrigerator and pipette 50ul of each extracted sample or control into the A-tubes containing Working Master Mix. Amplification must now be started within 45 minutes. Cap A-tubes tightly using capping tool. Move A-ring(s) to AREA 3 for amplification and detection on the Cobas Amplicor.

VIII. Quantification of Hepatitis C Viral RNA by Polymerase Chain Reaction by COBAS Amplicor HCV Monitor Test, v.2.1

Principle:

Serum for HCV quantification will be tested according to the guidelines of the package insert provided by Roche with the exception: separate document for HALT-C. The preparation, detection and interpretation of samples and controls will follow the protocol contained within the kits. The manufacture's recommendations for accepting valid runs and results will also be utilized.

According to the manufacture, dynamic range of the COBAS Amplicor HCV Monitor test for undiluted specimen is 600 – 500,000 IU/ml. The manufacturer recommends only one 1:100 dilution.

The COBAS Amplicor HCV Monitor test, v.2.0 is comprised of five major procedures: specimen preparation, reverse transcription of target RNA to generate cDNA, polymerase chain reaction (PCR), hybridization of the amplified product to a specific nucleic acid probe, and detection of the amplified product by color formation. The amplification and detection will be carried out in the COBAS Amplicor analyzer.

For a more detailed description of this assay, please refer to the COBAS AMPLICOR HCV Monitor Test v.2.0 package insert.

Turn around time:

HCV quantification run is set up as the amplification ring (a-ring) gets filled or as the results are needed. To run 1 A-ring, 9 patient specimens and 3 controls are required. If running 2 A-rings, 10 + 11 patient specimens and 3 controls are required.

Specimens:

Serum - See specimen requirements.

Equipment/Materials needed:

- HQUANT worksheet
- COBAS AMPLICOR A-Ring map
- COBAS AMPLICOR A-ring fitted with 12 A-tubes
- COBAS AMPLICOR A-ring holder
- Plastic resealable bag
- Eppendorf Repeater pipette with 1.25mL Combitip (sterile, individually wrapped)
- vortex
- 1.5 ml sterile screw cap tubes (Sarstedt)
- Tube racks
- 50 ml Falcon tubes
- Heat blocks, adjustable from ambient to 100°C
- Microcentrifuge with maximum rcf of 14,000 x g
- Precision pipettors (adjustable to deliver 1-20 µl, 20-200 µl, and 200-1000 µl)
- COBAS Amplicor HCV Monitor v. 2.0 Specimen preparation Kit
- COBAS Amplicor HCV Monitor v.2.0 controls kit
- COBAS Amplicor HCV Monitor v.2.0 Amplification kit
- COBAS Amplicor HCV Version v.2.0 Detection Kit
- COBAS Amplicor Detection Reagent Kit
- COBAS Amplicor Conjugate Detection Reagent
- COBAS Amplicor Wash Buffer
- Sterile transfer pipettes

COBAS Amplicor Analyzer
 PC with Amplilink
 Sterile 5mL serological pipette
 Pipetaid
 2L graduated cylinder

Reagents:

- a. Isopropyl alcohol - filter sterilize before use. Store at room temperature up to 6 months.
- b. 70% Ethanol
 Use fresh 70% ethanol for each assay - pour 35 mls ethanol (absolute) in a 50 ml conical tube, then add 15 mls of DEPC water. Mix before use. Store up to 24 hours.
- c. DEPC treated water
 Using a fume hood, add 100 µl diethyl pyrocarbonate (DEPC) per 100 ml deionized water. Mix for 1 hour at room temperature and filter through 0.2 µm filter. Store at 4°C for up to 6 months.
- d. HCV Probe suspension (CM4)
 In the product room, mix CM PS1 well by vortexing. Add 2.5 ml CM PS1 to one CM4 cassette. Place the cassette on the test specific reagent rack. Discard the open CM PS1 vial. Record the date of reagent prep on cassette with a red pen.
- e. HCV Quantitation Probe suspension (CQ4)
 In the product room, mix CQ PS1 well by vortexing. Add 2.5 ml CQ PS1 to one CQ4 cassette. Place the cassette on the test specific reagent rack. Discard the open CQ PS1 vial. Record the date of reagent prep on cassette with a red pen.
- f. Prepare the Working Substrate by pipetting 5 ml SB into one SB3 cassette in the product room. Pipette up and down to mix. Discard the open SB vial. This should be made fresh the day of detection. Record the date of prep on cassette.

Controls:

- a. Negative Control, HCV(-) C, is a kit negative synthetic RNA. One is run for each run.
- b. Low Positive Control, HCV L(+) C, is a kit low positive synthetic RNA. One is run for each run.
- c. High Positive Control, HCV H(+) C, is a kit high positive synthetic RNA. One is run for each run.
- d. Negative Human Plasma, NHP, is non-reactive for diluting various controls. Comes in kit.
- e. In-house negative Human Plasma, is negative plasma used to dilute patient specimens. This is acquired from HCV negative donors previously tested by HCV PCR test.

Procedure:

In each room, there is a 1-page procedure (see figure 2) taped to the wall near the work station. Please refer to this when performing the assay. For a more detailed procedure, please refer to the package insert.

There are dedicated rooms and equipment for each of the procedures. Move unidirectionally only from area 1 (clean room/reagent room) to area 2 (product room) when performing this assay. When aliquoting specimens, only open one tube at a time. Do not return unused specimen/reagent to the original container. Use aerosol resistant (plugged) tips when indicated to help prevent carry-over contamination.

Before proceeding, organize the tubes to match the worksheets and fill out the ring maps.

(see figure 1).

A. Reagent Preparation (PCR Hood in clean room)

1. Clean the PCR hood and pipettor with 70% ethanol.
2. Prepare working master mix by adding 100 µl of HCV Manganese Solution to one tube of HCV Master Mix. Recap the Master Mix tube and mix well by inverting the tube 10-15 times. The pink dye in the Manganese Solution is used for visual confirmation that the Manganese Solution has been added to the Master Mix. Discard remaining Manganese Solution.
3. Based on the worksheet, determine the appropriate number of A-rings needed for patient specimens and controls.
4. Place the A-ring(s) in the A-ring holder(s).
5. Pipette 50 µl of working master mix to each A-tube using a repeater pipette.
6. Place A-ring(s) containing working master mix in a resealable plastic bag. Seal securely and store at 2 to 8°C until use. This will only be **stable for 4 hours**.
7. Clean the PCR hood and pipettor used with 70% ethanol after each use. Turn on the UV light source for at least 15 minutes to decontaminate the area.

B. Sample Preparation (Chemical Fume Hood)

1. Warm Lysis reagent to 25 - 37°C and mix thoroughly to redissolve precipitate. Turn on heat block to 60°C.
2. Prepare working lysis reagent by adding 100 µl of HCV QS to the Lysis reagent and vortex. Discard remaining QS. The working lysis reagent is stable for 4 hours at room temperature. Make sure to keep the working lysis reagent separate from lysis reagent not containing QS.
3. Thaw serum or plasma specimens at room temperature and vortex for 3 to 5 seconds.
4. For each specimen or control, using a repeater, dispense 400 µl working lysis buffer into a 1.5 ml screw cap microfuge Sarstedt tube.
5. Prepare Controls as follows:
 - a. Vortex NHP, HCV(-)C, HCV L(+)C, HCV H(+)C for 5-10 sec.
 - b. Add 100ul NHP to each of the three control tubes. Cap tubes and vortex 3-5 seconds.
 - c. Add 100 µl of each control to its corresponding tubes and vortex 3-5 seconds.
6. Using a new plugged tip for each specimen, add 100 µl serum to each tube. Only open one tube at a time to avoid carry-over. Cap tubes and vortex well (3 - 5 seconds).

 If the sample requires a 1:100 dilution, take 10ul of a well mixed sample and add to 180ul of NHP or in-house negative plasma, vortex. Then take 20ul of diluted sample and add to 180ul NHP or in-house negative plasma. Vortex. Take 100ul of the last diluted sample into the Sarstedt tube containing the lysis reagent.
7. Incubate tubes for 10 minutes at 60°C heat block. Make sure to note the temperature on the worksheet.
8. Using a repeater, carefully add 500 µl isopropanol to each tube. Only open one tube at a time. Vortex well (3 to 5 seconds). Incubate at room temperature for 2 minutes.
9. Put an orientation mark on each tube and place into the microcentrifuge with the orientation mark facing outward, so that the pellet will align with the orientation mark. Centrifuge at maximum speed for 15 minutes.

10. Using a new transfer pipette for each specimen, carefully aspirate the supernatant without disturbing the pellet (which may not be visible). Be careful to remove as much supernatant as possible without disturbing the pellet. Discard the supernatant in a container containing bleach.
11. Opening one tube at a time, add 1 ml 70% ethanol using a repeater, re-cap, and vortex well (3 to 5 seconds).
12. Place the tubes in the microcentrifuge so that the pellet will align with the orientation mark. Centrifuge at maximum speed for 5 minutes.
13. Aspirate all liquid using a sterile transfer pipette. Do this without squeezing the bulb more than once. Place the tubes back in the rack without disturbing the tubes.
14. Go back through the tubes and aspirate again the liquid remaining inside the tubes with a sterile transfer pipette. Note: Residual ethanol will inhibit the QS and cause false negative or an "invalid QS" result.
15. Add 1 ml specimen diluent to each tube. Break apart the pellet as much as possible using a P200 pipettor fitted with a plugged tip. Vortex for 10 seconds. Analyze the processed specimens immediately or freeze the processed specimens at -70°C for up to one month, with no more than one freeze-thaw. Make note on the worksheet which freezer the extracted RNA is being stored in. More than one freeze-thaw cycle may result in loss of HCV QS RNA.
16. Add 50 μl of each processed specimen to the appropriate A-tube containing 50 μl master mix using a plugged tip. Be careful to avoid transferring any precipitated material that may not have been resuspended. Cap the A-tubes. Amplification must be started within 45 minutes of the time that the processed specimens and controls are added to the A-tubes containing master mix.

C. Reverse Transcription, Amplification, and Detection (PCR Product Room)

1. Maintenance of COBAS analyzer:
 - a. Turn on the COBAS analyzer and then activate Amplilink.
 - b. When prompted for user name, type in your initials.
 - c. Perform maintenance checks. Click on the service icon on Amplilink. Then click on the Services Actions tab. Examine each task and determine which service actions are due. Highlight the tasks that are due on the list and as you perform it, click on "update".

 For daily service, refer to the due date column for the next date to perform the daily actions.

 For weekly and 2 month service, the background of the service icon will turn yellow and eventually red. Maintenance should be performed when the icon is still yellow. Do not run any tests when this icon is red.

 After maintenance is complete, click on "update" to display the update service action entry dialogue box. The user may enter free text in the note box.
 - d. When system priming is required, in Amplilink click on "tools", then "prime". Click either normal or extend to begin priming. If new buffer is made, place the empty buffer bottle next to the reservoir so the next person will know the lot number of the wash buffer. Also initial and date on the tape on the carboy.
 - e. When the analyzer's syringes require changing, click on "tools", then "syringe exchange" to position the syringe plunger to the up position. Replace the syringe tips as outlined in the operator's manual and click on "yes" when asked if the

syringe tips are being replaced.

- f. Click on OK to record the servicing in the Service Log and to reset the counter in the Service Action folder. Make sure all the scheduled maintenance is done before leaving the service menu. **Transfer this information to the paper copy located in the blue binder.**

2. Instrument Loading and System Operation

For each A-ring used for Monitor assay, the following reagents and tests are required.

D-cups	74
Denaturing solution (DN)	12
Conjugate (CN)	72
Substrate (SB)	74
HCV Probe Suspension (CM4)	48
HCV QS Suspension (CQ4)	24
Amplicon Diluent (AD3)	36

- a. Examine the quantities of reagents on board the COBAS AMPLICOR analyzer as well as any open cassettes in the refrigerator. To determine how many tests are available in each of the cassettes, click on the “analyzer” and click on “cassettes”. Double click on the abbreviated reagent to display the expanded list. Click on the cassette barcode of interest and the “cassette status” will appear with expiration date, # of tests available, and when it first got placed on the COBAS analyzer.
- b. Prepare enough reagent cassettes to complete the workload. Refer to “reagents” for reagent preparation.
- c. Identify the reagent racks as generic or test specific using the keypad or barcode scanner on the COBAS analyzer as described in the Operator’s Manual for the COBAS AMPLICOR analyzer.
- d. Configure the reagent racks by inputting reagent positions and lot numbers into the instrument using the keypad or barcode scanner as described in the Operator’s Manual for the COBAS AMPLICOR. Do not print.
- e. Load the reagent rack onto the instrument using keypad or barcode scanner as described in the Operator’s Manual for the COBAS AMPLICOR analyzer. Make sure that each reagent cassette is in its assigned position and that each cassette fits tightly into its rack. Do not print.

As this information is saved on the analyzer, the racks and the cassettes should be displayed on the Amplilink screen. Be sure to take note the cassette colors. If the cassette is green, it indicates that the cassette is loaded and contains greater than 10 test counts. If the cassette is yellow, it indicates that the cassette is loaded and has expired or contains volume sufficient for 10 or less tests. If the cassette is red, there are 0 counts remaining or there is a volume mismatch. Discard any red cassettes.

- f. Discard any used up D-cup racks. Replace any empty D-cup racks with a new D-cup rack.
- g. Place the A-ring(s) into the thermal cycler segment(s) of the COBAS AMPLICOR analyzer.
- h. Load the A-ring(s) into the Analyzer using the keypad or barcode scanner as described in the Operator’s Manual for the COBAS AMPLICOR analyzer. If a previous run was run on “parallel” mode, remove the A-rings from TCA and/or TCB and place them in DP1 and/or DP2.

By clicking on the “analyzer” in Amplilink, the loaded A-rings should be displayed on screen.

- i. Create an A-ring Worklist by clicking on the “order” icon in Amplilink. First, click on the “+” button on top of the screen. This empties the summary list and lets you enter your own. Enter the A-ring ID. Click on the profile desired under “Profiles and Tests”. **MAKE SURE THE PROFILE MATCHES THE TESTS.** After selecting the profile, it will ask you for the QS# and control values. Click “accept” after editing. Enter the sample ID. When completed, **double check your work.** Then click “save”. The mnemonic for HCV quantification is **HCM**.
- j. 10. Make sure the COBAS analyzer is closed.
- k. 11. Press “start” in the status menu. It will then ask if you would like to be in parallel mode or basic mode. Note: parallel mode stops after the rings in TCA and TCB have completed the denaturation and DP1 and DP2 have completed the detection. Basic mode performs amplification and detection with stopping. Choose either parallel or basic, then press okay. The analyzer will then perform a load check to determine if it has all the reagents required for performing the tests ordered. If the load check passes, print the load check by clicking on the printer icon. Make sure the printed load check stays together with the worksheet and the A-ring map.

Results:

A. Result Calculation:

For each specimen and control, the COBAS AMPLICOR analyzer automatically determines the HCV RNA titer as follows. *HCV RNA titer is expressed in IU/ml.*

1. The COBAS instrument selects all HCV and Quantitation Standard amplicon dilutions that fall within the linear absorbance range of the assay (0.10-2.0 A_{660}). At least one HCV dilution and one QS amplicon dilution must fall within the linear range in order for a result to be calculated.
2. Corrects all selected absorbance values for background absorbance.
3. Determines that the A_{660} value for HCM QS is acceptable, confirming that the specimen processing, reverse transcription, amplification and detection steps were performed correctly.
4. Determines that all target dilutions of the HCV (-) C, yield A_{660} values ≤ 0.099 .
5. Determines Total HCV A_{660} and Total QS A_{660} for all selected dilutions and calculates the IU/ml for HCV L(+)C, and HCV H(+)C, and for each specimen using the maximum total absorbance calculated for the HCV and HCM QS amplicon and the number of IU/PCR assigned HCM QS.
6. Determines that the calculated IU/ml for HCV L(+)C, and HCV H(+)C, falls within the assigned range.
7. Generates a printout containing A_{660} values for all HCV and QS dilutions and the calculated HCV IU/ml for each specimen and control. The IU/ml is displayed in scientific notation under the specimen identification on the result printout.

B. Run Validation:

Check run printout for flags and comments to ensure that the run is valid. The run is **not** valid if any of the following flags or comments appear for the HCV MONITOR Controls.

Flag	Comment	Interpretation
Q	QS_INVALID	HCM QS A ₆₆₀ value for any control above or below acceptable range
N	NC_INVALID	HCV(-)C A ₆₆₀ above acceptable limit (>0.099)
H	HPC_INVALID	HCV H(+)C IU/ml value above or below assigned range
L	LPC_INVALID	HCV L(+)C IU/ml value above or below assigned range

If the run is invalid, repeat the entire test including specimen and control preparation, reverse transcription, amplification, and detection.

C. Data Review:

After the run is complete, click on the “results” icon on Amplilink, and then select the appropriate A-ring number. The results for that ring will be displayed on screen. It will ask if you would like to accept all or some of the results. Accept all results and print a copy of the results by clicking on the “printer” icon. Attach the results to the rest of your forms. The COBAS AMPLICOR analyzer provides a printout of the A₆₆₀ results together with the copy number calculated for each specimen and control. The A₆₆₀ values for the serial dilution of the target and QS amplicon should follow a pattern of decreasing A₆₆₀ values with increasing dilution factors, except for dilutions where the A₆₆₀ values exceed the linear range of the test (high titer samples) or for dilutions where the A₆₆₀ values approach background (low titer samples).

Check all results carefully. Normally, any value greater than **500,000 IU/ml** needs to be diluted 1:100 with negative serum and tested beginning with extraction. These results will **not** be flagged. However, for HALT-C testing, specimens should have already been tested diluted first.

HCV Amplicon Dilutions

HCV Dilutions 1, 2, 3, 4 represent neat, 1:8, 1:64 and 1:512 serial dilutions, respectively, of the HCV amplicon. The absorbance value should decrease with the serial dilutions, with the highest A₆₆₀ for each specimen or control in the first dilution (dilution 1) and the lowest A₆₆₀ in the last dilution (dilution 4). If the HCV A₆₆₀ values are not in sequence see *“Interpretation of results with out-of-sequence A₆₆₀ values”*.

HCV QS Amplicon Dilutions

HCV QS dilutions 1 and 2 represent neat and 1:8 serial dilutions, respectively, of the HCV QS amplicon. If the A₆₆₀ value for dilution 2 is greater than the A₆₆₀ value for dilution 1, then an error occurred. The result for that specimen is invalid. Repeat the entire test procedure (including specimen processing, amplification, and detection) for that sample.

D. Interpretation of results with out-of-sequence A₆₆₀ Values

1. The A₆₆₀ values for dilutions of the HCV amplicon should follow a pattern of decreasing A₆₆₀ values with increasing dilution factor, except for dilutions that are saturated and dilutions with background A₆₆₀ values.
2. In reactions containing high HCV RNA IU/ml, dilutions 1, 2, and 3 can become saturated, resulting in lowered A₆₆₀ results. These results are valid even though the HCV dilutions do not have decreasing A₆₆₀ values for dilutions 1-4.

3. In reactions containing low HCV RNA IU/ml, dilutions 2, 3, and 4 may contain background A_{660} values. These results are valid even though the HCV dilutions do not have decreasing A_{660} values for dilutions 1-4.
4. All dilutions with A_{660} values ≥ 0.1 and ≤ 2.0 should follow a pattern of decreasing A_{660} values for dilutions 1-4. **If A_{660} values ≥ 0.1 and ≤ 2.0 do not follow a pattern of decreasing A_{660} values for dilutions 1-4, then error occurred.** The results for this specimen or control are invalid. Repeat the entire test procedure (including specimen processing, amplification, and detection) for that sample.

E. Interpretation of Results:

For a valid run, check each individual specimen for flags or comments on the result print out. Interpret the results as follows:

1. A valid run may include both valid and invalid specimen results depending on whether flags and/or comments are obtained for the individual specimens.
2. Specimens with flags and/or comments are interpreted as follows:

Flag	Comment	Interpretation
Q	QS_INVALID	No QS dilution with A_{660} value within the linear absorbance range of the assay. The result for the specimen is invalid. Specimens with invalid results should be retested by repeating specimen and control preparation,
	TARGETOD_LO	A_{660} value for all HCV dilutions below the linear absorbance range of the assay. Report results as "HCV RNA not detected".
	TARGETOD_HI	A_{660} value for all HCV dilutions above the linear absorbance range of the assay. Report results as "Not Determined". If a quantitative result is required, dilute the original specimen 1:100 with HCV negative serum. Repeat the test and multiply reported result by 100.
	RESULT_LO	Calculated IU/ml are below the linear range of the assay. Report results as "HCV RNA detected, less than 600 HCV IU/ml".
	RESULT_HI	Calculated IU/ml are above the linear range of the assay. Report results as "greater than 850,000 HCV IU/ml". If a quantitative result is required, dilute the original specimen 1:100 with HCV negative serum. Repeat the test and multiply reported result by 100.
	TARG_RANGE	All target absorbance values are out-of-range for the assay. Report result as "Not Determined." If a quantitative result is required, the original specimen should be tested by repeating specimen preparation, reverse transcription, amplification, and detection.

F. Quantification Range:

The manufacture states the sensitivity of Roche Amplicor Monitor test v.2.0 to be 600 IU/ml. The range is set between 600 and 500,000 IU/ml. Any specimens greater than 500,000 IU/ml will be diluted 1:100 by performing 2 serial 1:10 dilutions.

G. Reporting results:

For HALT-C, if a run is valid, results above 800 IU/ml can be resulted without further confirmation. If a value is between 600 and 800 IU/ml, a positive qualitative result is required before this value can be resulted.

Quality Control:

1. At least one replicate of the AMPLICOR HCV (-) Control, the AMPLICOR HCV MONITOR Low (+) Control and the AMPLICOR HCV MONITOR High (+) Control must be included in each test run.
2. Check the run printout for flags and comments to ensure that the run is valid. Refer to the *Operator's Manual* for the COBAS AMPLICOR analyzer for printing and interpretation of flags and comments.
3. The AMPLICOR HCV (-) Control should yield a "Not Detected" result. If the HCV (-) Control is invalid, repeat all specimen and control preparation, reverse transcription, amplification, and detection. If the A_{660} of the HCV (-) Control is consistently greater than 0.099, contact your local Roche office for technical assistance.
4. The assigned range for the AMPLICOR HCV MONITOR Low (+) and High (+) Controls is specific for each test lot of control and is provided on the *COBAS AMPLICOR HCV MONITOR Data Card* supplied with the HCV MONITOR Controls Kit. These ranges are entered into the COBAS AMPLICOR analyzer using the keypad, barcode scanner or AMPLILINK software during the A-ring Worklist setup.

The value for both the HCV MONITOR Low (+) Control and High (+) Control should fall within the range indicated on the Data Card. If one or both controls do not meet criteria, the entire run is invalid. Repeat entire process (specimen and control preparation, reverse transcription, amplification, and detection). If the calculated HCV titer of one or both of the HCV MONITOR (+) Controls is consistently outside the assigned range, contact your local Roche office for technical assistance.

A. Saving Results

All accepted results should be saved. Each A-ring will be exported under the ring number into "e" hard drive of the computer in a "csv" format. The entire folder under HALT-C will then be copied to a disk and sent to NERI.

B. Miscellaneous

1. Once the results have been reviewed and validated by another technologist, the extracted RNA can be discarded.
2. Attached to each worksheet should be the a-ring map, load check from COBAS analyzer, and the results. All forms should be completed and initialed by the tech running the assay prior to review by the second technologist.
3. Make sure to clean up after yourselves and restock any empty cabinets.

Figures

Figure 1: COBAS AMPLICOR™ Monitor A-RING MAP

Figure 2: COBAS AMPLICOR™ HCV MONITOR TEST - VERSION 2.0

COBAS AMPLICOR™ Monitor A-RING MAP

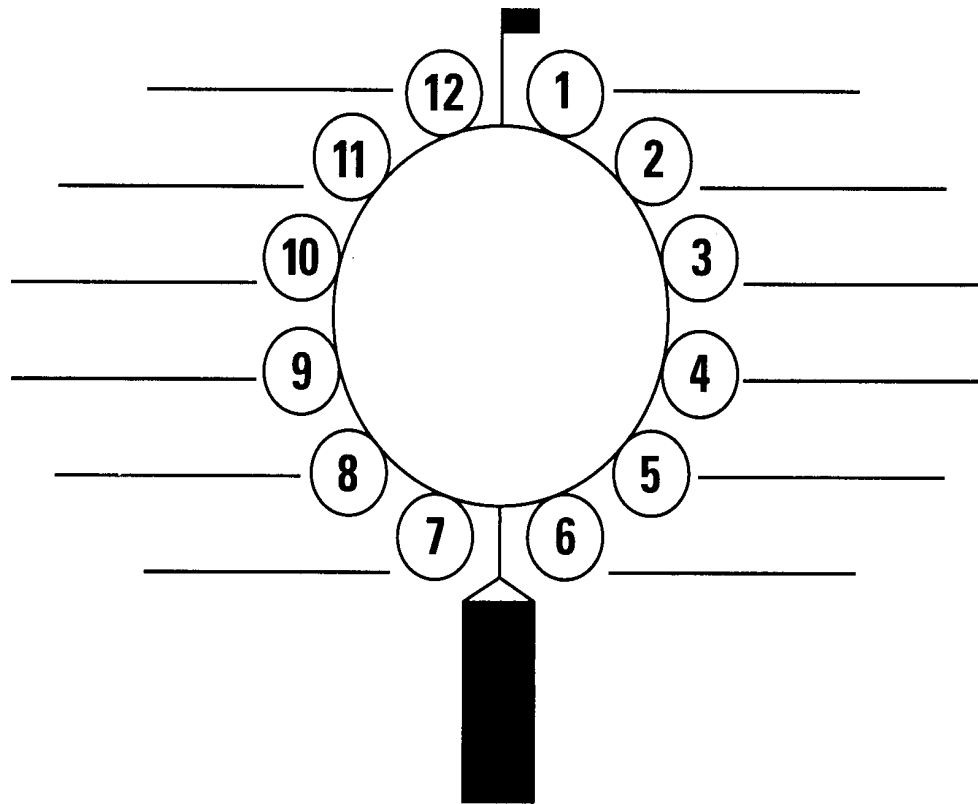
Ext. DATE: _____ Amp. & Detect: _____

OPERATOR: _____

Run #: _____

A-RING ID #: _____

Buffer Lot #: _____



Assay	Lot #	Exp. Date
HCV Monitor Kit		
QS IU's _____		
Refrigerator Temp (Clean Room) Refrig/Freezer Temp (Product Room) Specimen Freezer	On Chart? Y N Y N Y N	
Controls	Low range	High range
Low positive control		
High positive control		

Record Sample ID# next to each respective A-tube. Negative serum or NHP for Dilution: Lot #: _____

LOAD CHECK: Pass ☐ Fail ☐

Instrument QC OK (no error messages) ☐

If load check fails, STOP Transfer, correct problem, restart.

List any errors here: _____

COMMENTS:

COBAS AMPLICOR™ HCV MONITOR TEST - VERSION 2.0
For Research Use Only - Not For Use In Diagnostic Procedures

Cobas Amplicor™ PCR Reagent Preparation - AREA 1	Cobas Amplicor™ PCR Specimen Preparation AREA 2
<p>Always wear new gloves and lab coat when proceeding to next area. Amplification and detection kit must be at room temperature (RT).</p> <p><u>A. REAGENT PREPARATION</u></p> <ol style="list-style-type: none"> Determine the number of samples and controls to be tested. Vortex HCM Mn²⁺, v2.0 prior to HCM MMX, v2.0 preparation. Prepare the Working Master Mix by adding 100ul of HCM Mn²⁺, v2.0 to one vial of HCM MMX, v2.0. Mix by inverting 10-15 times. Add 50ul of Working Master Mix into each A-tube using a repeat pipettor. Do not cap the A-tubes at this time. Visually inspect A-tubes to ensure that the Working Master Mix has been added. Place the A-ring(s) in a resealable plastic bag and place the sealed bag in the refrigerator at 2-8°C in AREA 2 until ready to add samples. <p>NOTE: All VORTEXING SHOULD BE DONE FOR AT LEAST 3 SECONDS UNLESS OTHERWISE NOTED.</p>	<p><u>A. REAGENT PREPARATION</u></p> <ol style="list-style-type: none"> Prepare 70% ethanol by mixing 11.0 ml of 95% ethanol and 4.0 ml of deionized water. Make fresh daily. Visually inspect HCM Lysis Reagent (HCM LYS) to make sure that it is homogeneous and free of any precipitate. Warm between gloved hands and vortex. Vortex HCM QS v2.0 for 5-10 seconds. Prepare Working Lysis Reagent. Add 100ul of HCM QS v2.0 to one bottle of HCM LYS v2.0 and mix well. Note: Working Lysis Reagent is stable for 4 hours at RT. <p><u>B. SPECIMEN PREPARATION</u></p> <ol style="list-style-type: none"> Complete the A-ring Map for sample and control identifications. Determine the correct number of Sarstedt 1.5 ml tubes needed for samples and controls. Label and place an orientation mark on each tube. Add 400 µl of Working Lysis Reagent to each of the labeled tubes and cap the tubes. Vortex the negative human plasma (NHP), HCV (-)C, HCV L(+)C, HCV H(+), all v2.0 for 5-10 seconds Add 100ul of NHP to each of the three control tubes already containing 400ul of Working Lysis Reagent. Cap the tubes and vortex. Add 100ul of patient serum or plasma to the appropriate tube containing 400ul of Working Lysis Reagent and mix well by vortexing immediately. Add 100ul of HCV (-), L(+), and H(+) C, v2.0 to each of the respective tubes containing 400ul Working Lysis Reagent and mix well by vortexing immediately. Incubate all tubes in a dry heat block for 10 minutes at 60°C ± 2°C. Mix well by vortexing. Add 500ul of 100% isopropanol to each tube and vortex immediately. Incubate all tubes for 2 minutes at RT. Position the tubes in the centrifuge so that the orientation marks are facing outward. Centrifuge tubes for 15 minutes at ≥ 12,500 x g at RT. Using a fresh fine tip transfer pipette for each sample, carefully aspirate and discard the supernatant without disturbing the pellet (pellet may not be visible). <i>Perform only one aspiration!!</i> Add 1 ml of 70% ethanol to each tube and vortex. Centrifuge for 5 minutes at RT as above parameters. Using a fresh fine tip transfer pipette for each sample, carefully aspirate and discard the supernatant without disturbing the pellet (pellet should be clearly visible at this step). Repeat this process. <i>Two aspirations required!!</i> Resuspend pellet in 1 ml HCM diluent (DIL) v2.0 to each tube. Vigorously scrape/break pellet apart with a P200 pipettor fitted with a P200 plugged tip (new tip for each specimen). Vortex for 10 seconds. Start amplification within 3 hours of specimen and control preparation or store frozen at -70°C or colder for one month with no more than one freeze-thaw. Remove A-ring(s) from refrigerator and pipette 50ul of each extracted sample or control into the A-tubes containing Working Master Mix. Amplification must now be started within 45 minutes. Cap A-tubes tightly using capping tool. Move capped A-ring(s) to AREA 3 for amplification and detection on the Cobas Amplicor.