

UltraSensitive Roche Monitor Test, v1.5- Boom Extraction

1. PRINCIPLE:

1.1 Name and Intended Use

The AMPLICOR HIV-1 MONITOR test is an in vitro nucleic acid amplification test for the quantitation of Human Immunodeficiency Virus Type 1 (HIV-1) RNA in human plasma. The test is intended of use in conjunction with clinical presentation and other laboratory markers as an indicator of disease prognosis. This test is not intended to be used as a screening test for HIV or as a diagnostic test to confirm the presence of HIV infection.

1.2 Summary and Explanation of the Test

- 1.2.1 HIV is the etiologic agent of Acquired Immunodeficiency Syndrome (AIDS). The HIV infection can be transmitted by sexual contact, by exposure to blood or blood products, or by an infected mother to her fetus. HIV is a retrovirus. This class of virus relies upon reverse transcription, utilizing an enzyme called reverse transcriptase, to replicate viral particles.
- 1.2.2 There are six diagnostic methods currently in use to detect HIV infection: EIA for antibody detection, Western Blot for the detection of antibody directed against specific viral components, viral culture, EIA for HIV-1 antigen detection, indirect immunofluorescence assay, and radio immunoprecipitation assay. Most of these methods, however, do not provide a sensitive and specific method for the direct measurement of HIV-1 viral activity. The Polymerase Chain Reaction (PCR) technology provides a means to detect small amounts of viral nucleic acid with a high degree of specificity.
- 1.2.3 PCR allows the exponential amplification of a nucleic acid sequence. The small amounts of viral nucleic acid sequences may be released and reverse transcribed to provide nucleic acid sequence templates for amplification by PCR.

1.3 Biological Principles of the Procedure

- 1.3.1 The AMPLICOR HIV-1 MONITOR Test is based on five major processes: specimen preparation, reverse transcription (RT) of target RNA to generate cDNA, PCR amplification of target cDNA using HIV-1 specific complimentary primers, hybridization of the amplified products to oligonucleotide probes specific to the target(s), and detection of the probe – bound amplified products by colorimetric determinations.
- 1.3.2 The test permits the reverse transcription and amplification of HIV-1 and Quantitation Standard (QS) RNA to occur simultaneously. The Master Mix reagent contains a biotinylated primer pair specific for HIV-1 and QS target nucleic acid.

- 1.3.3 The quantitation of HIV-1 viral RNA is performed using as Quantitation Standard (QS). The QS is a noninfectious RNA transcript that contains the identical primer binding sites as the HIV-1 target and a unique probe binding region that allows QS amplicon to be distinguished from HIV-1 amplicon. The QS is incorporated into each individual specimen at a known copy number and is carried through the specimen preparation, reverse transcription, PCR amplification, hybridization, and detection steps along with the HIV-1 target and is amplified along with the HIV-1 target. HIV-1 RNA levels in the test specimens are determined by comparing the absorbance of the specimen to the absorbance obtained for the QS. Therefore, the QS compensates for any effects of inhibition and controls for the amplification process to allow the accurate quantitation of each specimen.

2. SPECIMEN PREPARATION

- 2.1 HIV-1 RNA is isolated from plasma by lysis of virus particles with a chaotropic agent followed by precipitation of the RNA with alcohol. A known number of QS RNA molecules is introduced into each specimen with the lysis reagent. The QS is carried through the specimen preparation, amplification, and detection steps and is used for the quantitation of HIV-1 RNA in the test specimen. The QS compensates for any effects of inhibition to permit the accurate quantitation of HIV-1 RNA in each specimen.

2.2 Boom Method

In the Boom method of purifying HIV-1 RNA from cells the chaotropic agent used is Guanidinium thiocyanate (GuSCN). GuSCN has been shown to be a powerful agent in the purification and detection of both DNA and RNA because of its potential to lyse cells combined with its potential to inactivate nucleases. In the presence of high concentrations of the chaotropic agent, GuSCN, HIV-1 RNA will bind to silica or glass particles. This method of extraction was developed to fulfill the following criteria. First, the method should be sensitive, reproducible, rapid, and simple, requiring no specialized equipment or specialized knowledge of biochemistry, thus allowing for nucleic acid purification from a large series of clinical specimens in a routine setting. Second, extracted nucleic acids should be sufficiently pure to allow for enzymatic modifications. Third, risks for personnel with regard to pathogens should be small. Finally, the chance of transmission of nucleic acids from sample to sample should be small. These demands have resulted in a highly standardized method for nucleic acid purification from human urine and serum.⁴ Specimens resulting in high negatives, those without a QS result, and those collected in anticoagulants other than EDTA and ACD are candidates for the Boom extraction method.

3. REVERSE TRANSCRIPTION AND PCR AMPLIFICATION

- 3.1 The AMPLICOR HIV-1 Test amplifies and detects a 142 base target sequence located in a highly conserved region of the HIV-1 gag gene, defined by the primers SK431 and SK462. The gag region encodes the group specific antigens or core structural proteins of the viron. The HIV-1 gag genes are generally about 1500 nucleotides in length and are located at the approximate positions 789-

2290 in the HIV genome. The reaction is performed with thermostable recombinant enzyme *Thermus thermophilus* DNA polymerase (*rTth* pol). In the presence of manganese and under the appropriate buffer condition, *rTth* pol has both reverse transcriptase (RT) and DNA polymerase activity. This allows both reverse transcription and PCR amplification to occur in the same reaction mixture.

3.2 Reverse Transcription

The processed specimens are added to the amplification mixture in reaction tubes in which both reverse transcription and PCR amplification occurs. The downstream or antisense primer (SK431) and the upstream or sense primer (SK462) are biotinylated at the 5' ends. The reaction mixture is heated to allow the downstream primer to anneal specifically to the HIV-1 and QS target RNA. In the presence of excess deoxynucleoside triphosphates (dNTPs), including deoxyadenosine, deoxyguanosine, deoxycytidine, deoxyuridine and thymidine triphosphates, *rTth* pol extends the annealed primer forming a complementary (cDNA) strand.

3.3 PCR Amplification

Following reverse transcription of the HIV-1 and QS target RNA, the reaction mixture is heated to denature the RNA:cDNA hybrid and expose the HIV-1 and QS target sequences. As the mixture cools, the upstream primer anneals to the cDNA strand, the *rTth* pol catalyzes the extension reaction, and a second DNA strand is synthesized. This completes the first cycle of PCR yielding a double stranded DNA copy (or amplicon) of each HIV-1 or QS RNA. The reaction mixture is heated again to separate the resulting double stranded DNA and expose the primer target sequences. As the mixture cools, the primers anneal to the target DNA. *rTth* pol, in the presence of excess dNTPs, extends the annealed primers along the target templated to produce a 142 base primer pair sequence termed an amplicon. Amplification occurs only in the region of the HIV-1 genome between the primers. The entire HIV-1 genome is not amplified.

3.4 Selective Amplification

Selective amplification of target nucleic acid from the clinical specimen in the AMPLICOR HIV-1 MONITOR Test is achieved by the use of AmpErase and deoxyuridine triphosphate (dUTP). AmpErase (uracil-N-glycosylase, UNG) recognizes and catalyzes the destruction of DNA strands containing deoxyuridine, but not DNA containing thymine. 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 contain deoxyuridine. Deoxyuridine renders contaminating amplicon susceptible to destruction by AmpErase prior to amplification of the target DNA. AmpErase, which is included in the Master Mix reagent, catalyzes the cleavage of deoxyuridine containing DNA at deoxyuridine residues by opening the deoxyribose chain at the C1 position. When heated in the first thermal cycling step at the alkaline pH of Master Mix, the amplicon DNA chain breaks at the position of the deoxyuridine, thereby rendering the DNA non-amplifiable. AmpErase is inactive at temperatures above 55°C; i.e. through out the thermal cycling steps and therefore does not destroy target amplicon. Following amplification, any residual enzyme is denatured by the addition of the denaturation solution, thereby preventing the degradation of target amplicon. AmpErase in the AMPLICOR HIV-1 MONITOR Test has

been demonstrated to inactivate 10 copies of deoxyuridine-containing HIV-1 amplicon per PCR.

4. HYBRIDIZATION REACTION

- 4.1 Following PCR amplification, the HIV-1 and QS amplicons are chemically denatured to form single stranded DNA by the addition of denaturation solution, and aliquots are added to separate wells of a microwell plate coated with HIV-1 specific (SK102) and QS-specific (CP35) oligonucleotide probes. HIV-1 and QS amplicons are bound to HIV-1 and QS wells, respectively, by hybridization to the microwell
- 4.2 Plate bound probes. To achieve quantitative results over a large dynamic range, serial dilutions of the denatured amplicon are analyzed in the microwell.

5. DETECTION REACTION

Following the hybridization reaction, the microwell plate is washed to remove unbound material and an avidin-horseradish peroxidase conjugate (Av-HRP) is added to each well of the plate. The AV-HRP binds to the biotin labeled amplicon captured by the plate bound oligonucleotide probes. The plate is washed again to remove unbound AV-HRP and a substrate solution containing hydrogen peroxide and 3,3',5,5'-tetramethylbenzidine (TMB) is added to the wells. In the presence of hydrogen peroxide, the bound horseradish peroxidase catalyzes the oxidation of TMB to form color complexes. The reaction is stopped by addition of a weak acid, and the optical density at 450nm is measured using an automated microwell plate reader.

6. HIV-1 RNA QUANTITATION

- 6.1 The AMPLICOR HIV-1 RNA MONITOR Test quantitates viral load by utilizing a second target sequence (QS) that is added to the amplification mixture at a known concentration. The QS is a non-infectious 219 base pair in vitro transcribed RNA molecule with primer binding region identical to those of the HIV-1 target sequence. The QS, therefore, contains SK431 and SK462 primer binding sites and generates a product of the same length (142 bases) and base composition as the HIV-1 target. The probe binding region of the QS was modified to differentiate QS-specific amplicon from HIV-1 target amplicon.
- 6.2 The optical density in each well of the plate is proportional to the amount of HIV-1 or QS amplicon in the well, and the total optical density is proportional to the amount of HIV-1 or QS RNA, respectively, input into each reverse transcription/PCR amplification reaction. The amount HIV-1 RNA in each specimen is calculated from the ratio of the total optical density for the HIV-1 specific well to the total optical density for the QS-specific well and the input number of QS RNA molecules using the following equation:

$$\frac{\text{Total HIV-1 OD}}{\text{Total QS OD}} \times \text{Input QS copies per PCR reaction} \times 4 = \text{HIV-1 RNA copies/mL}$$

7. SPECIMEN TYPES:

- 7.1 Plasma: This test is for use with plasma specimens. The minimum volume is a 2ml tube yielding 2 aliquots of 500 ul of plasma each. The ideal volume is a 7mL tube yielding 2 aliquots of 1ml each. EDTA, ACD, and Heparin anticoagulants are all acceptable.
- 7.2 Serum is acceptable for testing with this method. The minimum volume is a 2mL tube yielding 2 aliquots of 500 uL of plasma each. The ideal volume is a 7mL tube yielding 2 aliquots of 1mL each.
- 7.3 Urine and other body fluids are acceptable for testing with this method. The ideal volume is two aliquots of a minimum of 500uL of specimen in each tube.
- 7.4 **Standard (Universal) precautions will be observed for the collection, handling, transport and processing of all specimens.**

8. Handling Conditions:

Separate plasma from whole blood within 6 hours of collection by centrifugation at 800-1600 x g for 10 minutes at room temperature. The resulting plasma should then be spun again for an additional 10 minutes for clarification. Specimens should be stored at -80 degrees C. Roche in-house studies have shown that plasma specimens may be frozen and thawed up to three times. Studies have shown that cell-free plasma may be stored at 2 – 8 degrees C for up to 5 days. The Virology Subcommittee has approved the recommendation that bloods can be held up to 48 hours before processing. Each lab may have to validate this in their setting.

9. MATERIALS AND EQUIPMENT:

- 9.1 Materials:
 - 9.1.1 Boom Reagents:
 - 9.1.1.1 DEPC Distilled Water
 - 9.1.1.2 Trizma Base
 - 9.1.1.3 Concentrated HCl
 - 9.1.1.4 Guanidinium thiocyanate
 - 9.1.1.5 Triton-X 100
 - 9.1.1.6 Silicon dioxide
 - 9.1.2 Specimen Preparation Reagents:
 - 9.1.2.1 AMPLICOR HIV-1 MONITOR Lysis Reagent is not being used with the Boom method.
 - 9.1.2.2 AMPLICOR HIV-1 MONITOR Quantitation Standard
 - 9.1.2.3 AMPLICOR HIV-1 MONITOR Specimen Diluent
 - 9.1.2.4 Acetone
 - 9.1.2.5 70% Ethanol (**not denatured**), v/v with deionized water
 - 9.1.3 Control Reagents
 - 9.1.3.1 VQA 1.5 log 3 Control (1500 copies/mL) if testing ACTG patients
 - 9.1.3.2 Kit Controls (negative, low positive, high positive)
 - 9.1.4 Amplification Reagents
 - 9.1.4.1 AMPLICOR HIV-1 MONITOR Master Mix
 - 9.1.4.2 AMPLICOR HIV-1 MONITOR Manganese Solution
 - 9.1.5 Detection Reagents

- 9.1.5.1 MONITOR Denaturation Solution
 - 9.1.5.2 MONITOR Hybridization Buffer
 - 9.1.5.3 AMPLICOR Avidin-HRP Conjugate
 - 9.1.5.4 AMPLICOR Substrate A
 - 9.1.5.5 AMPLICOR Substrate B
 - 9.1.5.6 AMPLICOR Stop Reagent
 - 9.1.5.7 AMPLICOR 10X Wash Concentrate
 - 9.1.5.8 Distilled or deionized water
 - 9.1.5.9 AMPLICOR HIV-1 MONITOR Microwell Plate
- 9.2 Equipment:
- 9.2.1 Heraeus 17RS centrifuge
 - 9.2.2 Perkin Elmer GeneAmp® PCR system 9600 thermal cycler
 - 9.2.3 Consumables: tubes, caps, base, tray, and retainer
 - 9.2.4 Aerosol resistant pipette tips capable of holding 50 to 1000 microliters
 - 9.2.5 Pipettors, adjustable volume (20 – 200 µL), (50 µL), (200 µL)
 - 9.2.6 Impact Pipettor
 - 9.2.7 Narrow tip, sterile transfer pipettes
 - 9.2.8 Latex or nitrile gloves, powder-free
 - 9.2.9 Cryovials with caps, sterile, 2mL capacity
 - 9.2.10 Microwell plate sealers
 - 9.2.11 Disposable reagent reservoirs
 - 9.2.12 Disposable plastic bags
 - 9.2.13 Biological Safety Cabinet (BSC), Template Tamer Box, or equivalent
 - 9.2.14 Microplate Washer capable of washing a 96-well plate at 30 second intervals
 - 9.2.15 Microplate Reader with the following specifications: bandwidth = 10 ± 3 nm, absorbance range = 0 to a minimum of 2.00 when read at 450 angstroms, repeatability = 1% accuracy = 3% from 0 to 2.00 when read at 450 angstroms, drift <0.01 per hour
 - 9.2.16 Centrifuge
 - 9.2.17 Microcentrifuge
 - 9.2.18 Vortex Mixer
 - 9.2.19 Dry incubator 37°C($\pm 2^\circ\text{C}$)
 - 9.2.20 Personal computer with spreadsheet software
 - 9.2.21 Graduated cylinders, 100 to 1000 microliter capacities
 - 9.2.22 Sterile bottles
 - 9.2.23 Disposable pipettes, 5mL and 10mL
 - 9.2.24 Absorbent backed paper
 - 9.2.25 Disposable gown
 - 9.2.26 Clorox or equivalent
 - 9.2.27 Wescodyne, 10% solution
 - 9.2.28 Disposal troughs
 - 9.2.29 Biohazard boxes with lined red bags
 - 9.2.30 pH meter and calibrating buffer
 - 9.2.31 Dry heat block 56° ($\pm 2^\circ\text{C}$)

10. WARNINGS AND PRECAUTIONS:

- 10.1 For in Vitro diagnostic use.
- 10.2 Do not pipette by mouth.

- 10.3 Do not eat, drink, or smoke in laboratory work areas. Wear disposable gloves, laboratory coats and eye protection when handling specimens and kit reagents. Wash hands thoroughly after handling specimens and kit reagents.
- 10.4 Avoid microbial and ribonuclease contamination of reagents when removing aliquots from reagent bottles. The use of sterile disposable pipettes and pipette tips are recommended.
- 10.5 Do not pool reagents from different lots or from different bottles of the same lot.
- 10.6 Dispose of unused reagents and waste in accordance with country, federal, state and local regulations.
- 10.7 Do not use kit after expiration date.
- 10.8 Material Safety Data Sheets (MSDS) are available on request from Roche Response Center at 1-800-428-5030.
- 10.9 Workflow in the laboratory must proceed in a uni-directional manner, beginning in the Pre-Amplification Area and moving to the Post-Amplification Area. Pre-Amplification activities must begin with reagent preparation and proceed to specimen preparation. 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 removed before leaving that area. Equipment and supplies used for reagent preparation must not be used for specimen preparation activities or for pipetting or processing amplified DNA or other sources of target DNA. Post-Amplification supplies and equipment must be confined to the Post-Amplification Area at all times.
- 10.10 Specimens should be handled as if infectious using safe laboratory procedures. Thoroughly clean and disinfect all work surfaces with a freshly prepared solution of 0.5% sodium hypochlorite solution. A 1:10 dilution of most commercial bleaches will produce a 0.5% sodium hypochlorite solution.
- 10.11 This kit contains a component (NHP) derived from human blood. The source material has been assayed by the US FDA approved tests and found non-reactive for the presence of Hepatitis B Surface Antigen and antibodies to HIV-1-1/2 and HCV. No known test methods can offer complete assurance that product derived from human blood will not transmit infectious agents. Therefore, NHP should be handled as if infectious.
- 10.12 HIV-1 QS, HIV-1 DIL, HIV-1 MMX, HIV-1 Mn²⁺, HIV-1 low, and high positive control contain sodium azide. Sodium azide may react with lead and copper plumbing to form highly explosive metal azides. While disposing of sodium azide containing materials down laboratory sinks, flush the drains with large amounts of water to prevent azide buildup.
- 10.13 Wear eye protection, laboratory coats and disposable gloves when handling HIV-1 LYS, HIV-1 MMX, Monitor Denaturation, Monitor HYB, AV-HRP, SUB A, SUB B, Working substrate mixed, and Stop solution. Avoid contact of these materials

with the skin, eyes, or mucus membranes. If contact does occur, immediately wash with large amounts of water. Burns can occur if left untreated. If spills of these reagents occur, dilute with water before wiping dry.

- 10.14 SUB B and Working Substrate contain dimethylformamide, which has been reported to be toxic in high oral doses and may be harmful to the unborn child. Skin contact, inhalation of fumes and ingestion should be avoided. If skin contact occurs, wash thoroughly with soap and water and seek medical advice immediately.
- 10.15 Do not allow HIV-1 LYS, which contains guanidine thiocyanate, to contact sodium hypochlorite solution. This mixture can produce a highly toxic gas.
- 10.16 Screw-cap tubes must be used for specimen and control preparation to prevent splashing and potential cross-contamination of specimens. Do not use snap cap tubes.

11. PREPARATION OF BOOM REAGENTS:

- 11.1 0.2 M EDTA, pH 8.0: Add 9g EDTA to 121LI DEPC distilled water. Mix well. The EDTA takes a long time to go into solution. Check pH. Adjust to pH 8.0. This mixture is for preparing the L6 Lysis Buffer Preparation. Store at room temperature for 6 months.
- 11.2 L2 Buffer: Dissolve 12.11g Trizma base in 800mL DEPC distilled water. Adjust pH to 6.4 with concentrated HCl (~7.75mL). Allow to cool to room temperature. Check pH and readjust to pH 6.4, if necessary. Add DEPC distilled water to 1000mL. This mixture is for preparing the L2 Wash Buffer and L6 Lysis Buffer. Store at room temperature in the dark for 6 months.
- 11.3 L2 Wash Buffer: Dissolve 300g Guanidinium thiocyanate (GuSCN) in 250mL of L2 Buffer. Heating at 60-65°C with shaking or stirring facilitates this process. Store at room temperature in the dark for 6 months.
- 11.4 L6 Lysis Buffer: Dissolve 330g GuSCN in 275mL of L2 Buffer (**NOT L2 Wash Buffer**). Heating at 60-65°C with shaking or stirring facilitates this process. Add 60.5ml of 0.2M EDTA, pH 8.0. Add 7.15g of Triton-X 100. Mix to dissolve. Store at room temperature in the dark for 6 months.
- 11.5 Silica Reagent: Place 60g of Silicon dioxide in a 500ml glass cylinder. Add dH₂O to 500mL and allow to stand overnight at room temperature in the dark. Remove supernatant (~430ml) by suction. Add dH₂O to 500ml and shake vigorously to resuspend silica. Let stand at least 5 hours at room temperature. Remove supernatant (~440mL) by suction. Adjust pH to 2 with ~400µL of concentrated HCl. Dispense in smaller glass containers and autoclave for 15 minutes. Store at room temperature in the dark for 6 months.

12. SPECIMEN PREPARATION REAGENTS AMPLICOR HIV-1 MONITOR:

- 12.1 AMPLICOR HIV-1 MONITOR Lysis Reagent is not being used in this procedure. L6 Buffer is used in its place.
- 12.2 AMPLICOR HIV-1 MONITOR Quantitation Standard (QS): A buffered solution containing Quantitation standard RNA, poly rA RNA, Tris-HCl, EDTA, and 0.05% Sodium azide. Store at 2 to 8°C until expiration date.
- 12.3 Working Lysis Reagent: Add 25µL of the QS to one bottle of Lysis reagent. Mix thoroughly. The pink dye is used as a visual confirmation that the QS has been added to the lysis reagent. Discard remaining QS. Store at room temperature and use within 4 hours.
- 12.4 AMPLICOR HIV-1 MONITOR Specimen Diluent: A buffered solution containing Tris-HCl, EDTA, poly rA RNA, and 0.05% sodium azide. Store at 2 to 8°C until expiration date.
- 12.5 Isopropanol(2,2 – propanol): Store at room temperature in the flammable liquids cabinet.
- 12.6 70% Ethanol (**not denatured**), v/v with deionized water: Dilute absolute ethyl alcohol with distilled, deionized water to a 70%(Volume depends on the grade of ethanol-95% to 100%). Store at room temperature in flammable liquids cabinet. Prepare fresh 70% daily.

13. CONTROL REAGENTS:

- 13.1 VQA Controls: A solution containing positive control supernatant spiked into seronegative plasma at varying concentrations. Store at –70°C until expiration date.
- 13.2 Kit Controls (For non-ACTG samples) including NHP, HIV-1(-)C, HIV-1L(+)C, HIV-1H(+)C

14. AMPLIFICATION REAGENTS:

- 14.1 AMPLICOR HIV-1 MONITOR Master Mix: A bicine buffered solution containing <30% glycerol, potassium acetate, <0.001% dATP, dCTP, TTP, dUTP, biotinylated primers, <0.01%r *Tth* Pol, <0.01% AmpErase and 0.05% sodium azide. Store at 2 to 8°C until expiration date.
- 14.2 AMPLICOR HIV-1 MONITOR Manganese Solution: A solution containing manganese acetate and 0.05% sodium azide. Store at 2 to 8°C until expiration date.
- 14.3 Working Master Mix: Add 100µL of AMPLICOR HIV-1 MONITOR Manganese solution to one tube of AMPLICOR HIV-1 MONITOR Master Mix. It is not necessary to measure the volume of Master Mix. Recap the Master Mix tube and mix well by inverting the tube 10 – 15 times or by mixing with a vortex for 3 – 5 seconds. The pink dye is used for visual confirmation that the Manganese

solution has been added. Discard the remaining Manganese Solution. Working Master Mix should be stored at 2 - 8°C and use within 4 hours.

15. DETECTION REAGENTS:

- 15.1 MONITOR Denaturation Solution: A solution of EDTA, 1.6% sodium hydroxide, and amaranth dye. Store at 2 to 25°C until expiration date.
- 15.2 MONITOR Hybridization Buffer: A sodium phosphate solution containing <0.2% solubilizer and <25% chaotrope. Store at 2 to 25°C until expiration date.
- 15.3 AMPLICOR Avidin-HRP Conjugate: An avidin-horseradish peroxidase conjugate in a buffered solution containing 1% ProClin 150™, emulsifier, bovine gamma globulin and 0.1% phenol. Store at 2 to 8°C until expiration date. Once opened these reagents are stable for 3 months or the expiration date, whichever comes first.
- 15.4 AMPLICOR Substrate A: A citrate solution containing 0.01% H₂O₂ and 0.1% ProClin 150. Store at 2 to 8°C until expiration date. Once opened these reagents are stable for 3 months or the expiration date, whichever comes first. Do not expose to metals, oxidizing agents or direct light.
- 15.5 AMPLICOR Substrate B: Contains 0.1% 3,3',5,5'-tetramethylbenzidine in 40% dimethylformamide. Store at 2 to 8°C until expiration date. Once opened these reagents are stable for 3 months or the expiration date, whichever comes first. Do not expose to metals, oxidizing agents or direct light.
- 15.6 Working Substrate Solution: For each microplate, mix 12mL of Substrate A with 3ml of Substrate B. Protect from light. Store at room temperature. Use within 3 hours. Do not expose to metals, oxidizing agents or direct light.
- 15.7 AMPLICOR Stop Reagent: Contains 4.9% sulfuric acid. Store at 2 to 25°C until expiration date.
- 15.8 AMPLICOR 10X Wash Concentrate: A sodium phosphate and sodium salt solution containing EDTA, <2% detergent and 0.5% ProClin 300. Store at 2 to 25°C until expiration date.
- 15.9 Wash Buffer 1X:
 - 15.9.1 Dissolve any precipitate by warming to 30 to 37°C.
 - 15.9.2 Add 100ml of 10X wash concentrate to 900mL of distilled, deionized water.
 - 15.9.3 Mix well. Store in a clean, closed container at 2 to 25°C for up to 2 weeks. Label with preparation and expiration dates.
- 15.10 AMPLICOR HIV-1 MONITOR Microwell Plate: An oligonucleotide probe coated microwell plate with twelve 8 well strips in one resealable pouch with desiccant,

HIV-specific DNA probe (rows A-F), Quantitation Standard specific DNA probe (rows G-H). Store at 2 to 8°C in the foil pouch. The plate is stable in an unopened pouch until the expiration date. Once opened the plate is stable for 3 months or until expiration date, whichever comes first) as long as it is store in the resealable pouch.

16. CALIBRATION: None

17. QUALITY CONTROL:

- 17.1 Three kit controls must be included in every run
- 17.2 A run contains a maximum of four plates
- 17.3 One external VQA control ($1.5 \log_{10} 3$) must be included in every run
- 17.4 A minimum of one control must be included on each plate within a run
- 17.5 As with any new laboratory procedure, new operators should consider the use of additional controls until such a time as high degree of confidence is reached in their ability to perform the test correctly.
- 17.6 All controls and patient specimens should yield OD values for the QS that meet the criteria described in the Results section, demonstrating that the specimen processing, reverse transcription, amplification, and detection steps were performed correctly. If any specimen has a QS OD value that does not meet the criteria described above, the result for that specimen is invalid, but the run is still acceptable.
- 17.7 Basic Criteria to define a valid run for the Roche HIV-1 Monitor™ Test
 - 17.7.1 All kit copy control results must be valid
 - 17.7.2 No out of sequence errors in the 0.2-2.0 working OD range
 - 17.7.3 QS result must be valid- OD must be between 0.3-2.0 in 1:1 or 1:5 dilution
 - 17.7.4 No OD ratio failures (run may be considered valid after re-detection of control)
 - 17.7.5 Any failure of the control criteria results in an invalid run. Re-detection of one or all controls may validate a run, otherwise, all samples and controls need to be re-extracted, re-amplified, and re-detected.
 - 17.7.6 All Kit positive control results must be within the range provided by the manufacturer
 - 17.7.6.1 if outside range, all samples within the run need to be repeated
 - 17.7.6.2 control can be re-detected in cases where applicable

17.7.7 Kit negative control must be undetectable- all WT ODs <0.2

17.7.8 The VQA will provide 3 standard deviation ranges for each lot of VQA controls

17.8 For the ultra-sensitive Roche HIV-1 Monitor™ Test, the external VQA control must be within 3SD range provided

17.8.1 if outside range, all samples within the batch need to be repeated

17.8.2 control can be redetected in cases where applicable

Table 1: Theoretical Range for Demonstrative Purposes

VQA CC	Assay	Assay SD Log ₁₀ 10 units ¹⁰	-3SD	-2SD	-1SD	Median	+1SD	+2SD	+3SD
1.5 log ₁₀ 3	Ultra-Sensitive	0.166	476	698	1023	1500	2198	3221	4721

Validity criteria have been established using the Roche HIV-1 Monitor™ package insert as well as VQA data. VQA analyses demonstrate that the out of sequence rule should only be applied to ODs that fall within the 0.2-2.0 working range. Additional studies demonstrate that the OD ratio rule can predict accuracy in estimations of RNA recovery. The OD ratio may be obtained in situations where there are two or more WT ODs between 0.2-2.0. The background OD (0.07) is first subtracted from the OD values, and then a ratio of the higher OD to the lower OD is determined. The OD ratio is deemed acceptable if the OD ratio is between 3-7 fold. VQA analyses demonstrate that OD ratios that fall outside this range may result in an inaccurate estimation of RNA copy number.

18. TROUBLESHOOTING/MAINTENANCE:

18.1 Maintenance is to be performed on the plate washer, reader, and thermal cycler as described in the maintenance manual. To troubleshoot the thermal cycler, check the Perkin Elmer handbook.

18.2 To troubleshoot any problems with the assay itself, please call Roche PCR technical support at 1-800-428-5030.

19. PROCEDURE- STEPWISE:

19.1 Preliminary Statements

19.1.1 All reagents must be at ambient temperature (18 to 24°C) before using them. All pipette tips used in this procedure must be aerosol resistant (i.e., plugged). All reagents should be visually examined for sufficient reagent volume before beginning the test procedure.

- 19.1.2 Workflow in the laboratory should proceed in a uni-directional manner, beginning in the Reagent Preparation Area and moving to the Specimen Preparation Area and then to the Amplification/Detection Area. Pre-amplification activities must begin with reagent preparation and proceed to specimen preparation. If using the same area for reagent preparation and specimen preparation, the area must be cleaned in between and the germicidal light used for at least one half hour prior to use. Ideally these two operations should be performed in two separate areas. Supplies and equipment must be dedicated to each activity and not used for pipetting or processing amplified DNA or other sources of target DNA. Amplification and detection supplies and equipment must be confined to the Amplification/Detection area at all times.
- 19.1.3 Run Size (hospital patients): Each kit contains sufficient reagents for two 12 test batches, which may be performed separately or simultaneously. If the available kits are the same lot number, up to three plates at a time may be performed. This may be difficult to complete in an 8 hour period due to extra processing for the Boom method. It is required that one of each VQA control or kit control be included in each run.
- 19.1.4 Workflow: The AMPLICOR HIV-1 MONITOR Test can be completed in one day or over two days. If the testing is to be done in a single day, follow the instructions in order. If the testing is to be completed in 2 days the procedure may be stopped after specimen preparation or after amplification. To perform the specimen processing on day one and amplification/detection on day two, begin with section B through the step where you will freeze the samples at -20°C until amplification can occur. On day 2 begin with section A, thaw the processed specimens at room temperature and then continue with step 18 in section B. To complete specimen preparation and amplification on day 1 and detection on day 2, perform sections A, B, C on day 1 and store the denatured amplicon at 2 to 8°C for up to one week. Continue with section D on day 2.
- 19.2 Reagent Preparation
- 19.2.1 Preparation working Master Mix by adding 100 microliters of Manganese Solution to one tube of Master Mix. Recap the Master Mix tube and mix well by inverting the tube 10-15 times (this is “working master mix”). The pink dye in the Manganese Solution is for visual confirmation that the Manganese Solution has been added to the Master Mix. Leftover Manganese Solution should be discarded.
- 19.2.2 Place the appropriate number of PCR reaction tubes, 12 tubes to a row, into a MicroAmp sample tray and lock the tubes in position with the tube retainer.
- 19.2.3 Pipette 50 microliters of working master mix into each PCR tube using a micropipettor with an aerosol resistant tip. Discard leftover working master mix.

19.2.4 Place the microtube tray in a plastic zip-lock bag and store the tray at 4°C until the specimen preparation is completed. **Amplification must begin within 4 hours of the preparation of the working master mix.**

19.2.5 If performing the specimen preparation in the same area, remember to clean the area and put on the germicidal light for at least one half an hour.

19.3 Specimen and Control Preparation

19.3.1 Thaw specimens and controls to room temperature. Vortex well.

19.3.2 Pre-cool the Heraeus 17RS centrifuge to 4 degrees C. Aliquot 0.5mL of specimen or VQA control to a labeled conical microcentrifuge tube. If processing kit controls aliquot 0.5ml NHP into control tubes. Spin the specimens in the Heraeus centrifuge, using rotor HFA 22.1, at 17,000 rpm for 1 hour at 4 degrees C.

19.3.3 After centrifugation, take off supernatant leaving approximately 25uL and pellet behind. Discard supernatant.

19.3.4 For each kit control add 12.5uL of the appropriate controls to the labeled control tubes.

19.3.5 Add 25µL of Roche QS to 12mL of L6 Lysis Buffer.

19.3.6 Mix well by vortexing for 5 seconds and tilting tube several times.

19.3.7 Aliquot 900µL of L6 Lysis Buffer + QS into each specimen and control tube.

19.3.8 Resuspend silica solution by vigorous mixing.

19.3.9 Add 40µL of silica to each specimen and control tube. Vortex until silica is resuspended.

19.3.10 Incubate at room temperature for 10 minutes. Vortex each tube for 5 seconds.

19.3.11 Centrifuge for 15 seconds at 12,000 x g. Aspirate supernatant with fine-tipped transfer pipette and discard.

19.3.12 Wash Step 1:

19.3.12.1 Add 1.0mL of **L2 Wash Buffer** and vortex until pellet is completely resuspended.

19.3.12.2 Centrifuge for 15 seconds at 12,000 x g.

19.3.12.3 Aspirate supernatant.

19.3.13 Wash Step 2: Repeat Step 19.3.12

19.3.14 Wash Step 3:

19.3.14.1 Add 1.0mL of 70% Ethanol and vortex until pellet is completely resuspended.

19.3.14.2 Centrifuge for 15 seconds at 12,000 x g.

19.3.14.3 Aspirate supernatant.

19.3.15 Wash Step 4: Repeat Step 19.3.14

19.3.16 Wash Step 5:

19.3.16.1 Add 1.0mL Acetone and vortex until pellet is completely resuspended.

19.3.16.2 Centrifuge for 15 seconds at 12,000 x g.

19.3.16.3 Aspirate supernatant.

19.3.17 Evaporate acetone by incubating open tubes at 56°C for 10-15 minutes. Pellets should be dry.

19.3.18 Add 100µL of Sample Diluent (Roche Monitor kit) to each tube. Vortex until pellet is resuspended.

19.3.19 Incubate at 10 minutes at 56°C. Vortex for 5 seconds.

19.3.20 Centrifuge for 2 minutes at 12,000 x g to pellet silica. The supernatant contains the RNA.

19.3.21 Add 50µL to each microwell containing 50µl of Master Mix. Amplify immediately.

19.3.22 If not amplifying immediately do not add to microwells containing Master Mix. Store frozen at -20°C until ready to proceed with the HIV Monitor Assay. If samples are frozen prior to amplification, thaw them, vortex to resuspend the silica, heat at 56°C for 10 minutes, and centrifuge for 2 minutes at 12,000 x g before adding to microwells.

19.3.23 Decontaminate work area with a 1:10 dilution of bleach made fresh that day. Follow by cleaning area with propanol.

19.4 Reverse Transcription and Amplification

19.4.1 **NOTE: Turn on the GeneAmp PCR System 9600 thermal cycler at least 30 minutes prior to beginning amplification.**

- 19.4.2 Place the reaction tray into the thermal cycler sample block. **Make sure that the notch in the reaction tray is at the left of the block, and that the rim of the tray is seated in the channel around the block.**
 - 19.4.3 Make certain that the cover knob is turned completely counterclockwise and slide the cover forward.
 - 19.4.4 **Turn the cover knob clockwise until hand tight.**
 - 19.4.5 Program the GeneAmp System 9600 thermal cycler as follows:

Hold	2 minutes at 50°C
Hold	30 minutes at 60°C
4 cycles	10 seconds at 95°C, 10 seconds at 55°C, 10 seconds at 72°C
26 cycles	10 seconds at 90°C, 10 seconds at 60°C, 10 seconds at 72°C
Hold	15 minutes at 72°C
 - 19.4.6 In the CYCLE programs the ramp time and allowed setpoint error should be left at the default settings. Link the 5 programs together into a METHOD program. On our thermal cycler is **METHOD 10**.
 - 19.4.7 Start the method program. The program runs for approximately one hour and 30 minutes.
 - 19.4.8 Remove the reaction tray from the thermal cycler beyond the end of the final Hold period. Do not allow the tubes to remain in the thermal cycler beyond the end of the final Hold period. **Do not bring amplified DNA into the other areas. The amplified material should be considered to be significant potential source of DNA contamination.**
 - 19.4.9 Remove the caps from the reaction tubes carefully so as to avoid aerosols of the amplification products. Immediately pipette 100µl of MONITOR Denaturation Solution into each reaction tube using a multichannel Pipettor, and mix carefully pipetting up and down at least 5 times. Preferably use the AMPLICOR Electronic IMPACT Pipettor set on Program 1 (see separate procedure).
 - 19.4.10 The detection amplicon can be held at room temperature no more than 2 hours before proceeding to the detection reaction. If the detection reaction can not be performed within this time, re-cap the tubes and store the denatured amplicons at 2 to 8°C for up to one week.
- 19.5 Detection
- 19.5.1 Warm all reagents and amplicons to room temperature prior to proceeding with the detection.
 - 19.5.2 Prepare a sufficient amount of working Wash Solution (dilute 1 part of the 10X with 9 parts of distilled or deionized water). This working Wash Solution is stable for up to 2 weeks at room temperature.

- 19.5.3 Allow the microwell detection plate to warm to room temperature before removing it from its foil pouch.
- 19.5.4 Add 100 μ L of MONITOR Hybridization Buffer to each well using Program 2 on the IMPACT Pipettor.
- 19.5.5 Add 25 μ L of the denatured amplicons to the wells of row A of the detection plate, mix up and down 10 times (twice). Use aerosol resistant plugged pipette tips. Make serial 5-fold dilutions in wells B through F as follows: transfer 25 μ L from row A to B and mix as before. Continue through row F. Mix row F as before, then remove and discard 25 μ L. Discard pipette tips. This may be done using the IMPACT Pipettor Program 3 two times, where 25 μ L is transferred and mixed by pipetting 60 μ L up and down 10 times, and aspirates 25 μ L.
- 19.5.6 Add 25 μ L of the denatured amplicons to the wells of row G of the detection plate in the same manner as described in step 5. Mix as described in step 5 and transfer 25 μ L from row G to row H, again, mix as described in step 5; remove and discard 25 μ L from row H along with the pipette tips.
- 19.5.7 Cover the plate and incubate it for 1 hour at 37°C (\pm 2°C).
- 19.5.8 Wash the entire detection plate 5 times with the working Wash Solution and an automated microplate washer. The microplate washer should entirely fill each well (400-450 μ L), allow each row (or column) soak for 30 seconds, then aspirate the entire contents of each well before proceeding to the next cycle.
- 19.5.9 Add 100 μ L of Avidin-HRP conjugate to each well, cover plate and incubate for 15 minutes at 37°C (\pm 2°C).
- 19.5.10 Wash the plate as described in step 8.
- 19.5.11 Prepare the working Substrate solution by mixing 4 parts of substrate A with 1 part of substrate B. Protect the working solution from direct light.
- 19.5.12 Pipette 100 μ L of the working Substrate solution to each well and allow the color (light blue to dark blue) to develop for 10 minutes at room temperature in the dark.
- 19.5.13 Add 100 μ L of Stop Reagent to each well (blue color will turn to a yellow color).
- 19.5.14 Measure the optical density at 450 angstroms within 10 minutes of adding the Stop Reagent otherwise less dilute wells will precipitate resulting in a lower OD.
- 19.5.15 Decontaminate work area with a 1:10 bleach solution made fresh that day.

20. CALCULATIONS:

20.1 Manual Calculations

20.1.1 For each specimen, control, or standard, choose the appropriate HIV well, as follows:

20.1.1.1 The HIV wells in rows A through F represent neat and 5-, 25-, 125-, 625-, and 3125-fold serial dilutions of the amplicons, respectively. The absorbance values should decrease with the serial dilutions, with the highest value for each test in row A and the lowest value in row F.

20.1.1.2 Choose the well where the raw OD is in the range of 0.200 to 2.0 OD units. If more than 1 well is in this range, choose the well with the larger dilution factor (i.e., the smaller OD).

20.1.1.3 If any of the following conditions exist see Unexpected Results, below: all HIV OD values <0.200; all HIV OD values >2.0; HIV OD values are not in sequence (i.e., the OD values do not increase from well A to well F).

20.1.2 Subtract a background value of 0.070 OD units from each of the selected HIV OD values.

20.1.3 Calculate the "total HIV OD" by multiplying the value derived in step 20.1.2 by the dilution factor associated with that well.

20.1.4 For each specimen, control, or standard, choose the appropriate QS well, as follows:

20.1.4.1 The QS wells in rows G and H represent neat and 5-fold dilutions of the amplification products, respectively. The absorbance value in row G should be greater than the value in row H.

20.1.4.2 Choose the well where the OD is in the range of 0.300 to 2.0 OD units. If both wells are in this range, choose well H.

20.1.4.3 If one of the following conditions exist, see Unexpected Results, below: both QS OD values <0.300; both QS OD values >2.0: QS OD values are not in sequence (i.e., the OD values do not decrease from well G to well H).

20.1.5 Subtract a background value of 0.070 OD units from each of the selected QS OD values.

20.1.6 Calculate the "total QS OD" by multiplying the value derived in step 20.1.5 by the dilution factor associated with that well.

20.1.7 Calculate HIV-1 RNA Copies/mL plasma as follows:

Total HIV-1 OD

----- X Input QS copies per PCR reaction X 4 = HIV-1 RNA copies/ml
Total QS OD

20.2 Computerized (Manual – LDMS) Calculations: Follow the instructions for LDMS use in the LDMS manual

21. RESULT CRITERIA:

- 21.1 If all of the HIV wells have OD values less than 0.200, but the QS wells have the expected values, use 0.200 as the HIV OD, calculate the result, and report the result as “Not detected, less than” the calculated value.
- 21.2 If all the HIV wells have OD values greater than 2.0, but the QS wells have the expected values, either an error occurred in the test, or the HIV copy number is above the dynamic range of the assay. Report the result as “Not determined”. Repeat the entire assay, making a 1:50 dilution with HIV negative human plasma. Calculate the results as above and multiply by 50.
- 21.3 If the HIV wells do not follow the pattern of decreasing OD values from well A to well F, an error in dilution may have occurred. Examine the data according to the following criteria to determine if an error occurred. If an error occurred, report the result the as “Not determined” and repeat the entire assay including specimen preparation; otherwise, calculate and report the result as described above:
- 21.3.1 The OD values for HIV wells should follow a pattern of decreasing OD values with increasing Dilution Factor 9 (i.e., from well A to F), except for well that are saturated and wells with background OD values.
- 21.3.2 In reactions containing high HIV-1 RNA copies per ml, wells A, B, and C can become saturated turning a greenish – brown color prior to the addition of Stop Solution and a brown color after addition of Stop Solution, resulting in lower OD. These results are valid even though the HIV wells do not have decreasing OD values from wells A through F.
- 21.3.3 In reactions containing low HIV-1 RNA copies per ml, wells B through F may contain background OD values. Such tests are valid even though the HIV wells do not have decreasing OD values from well A through F. Wells with OD values (>2.0) may be saturated and wells with very low OD values (<0.2) are close to background. These wells may not follow a pattern of decreasing OD values from well A to well F.
- 21.3.4 All wells with OD values ≤ 2.0 and ≥ 0.2 should follow a pattern of decreasing OD values from well A to well F. If OD values do not follow a pattern of decreasing OD values from A to F then an error occurred.

- 21.4 If both QS wells have OD values less than 0.300, either the processed sample was inhibitory to the amplification, or the RNA was not recovered from the sample. The result for that specimen is invalid. Repeat the entire test procedure including specimen processing.
- 21.5 If both QS wells have OD values greater 2.0, an error occurred. The result for that specimen is invalid. Repeat the entire test procedure including specimen processing.
- 21.6 If the absorbance well H is greater than the absorbance of well G, and error occurred. The result for that specimen is invalid. Repeat the entire test procedure including specimen processing.
- 21.7 OD dilutions in the 0.2 – 2.0 working range that are less than 3 fold or greater than 7 fold must be re-detected.
- 21.8 Undetectable samples greater than the cutoff (ultra assay is 50) re-extracting, re-amplifying, and re-detecting.
- 21.9 Any out of sequence errors in the 0.2 – 2.0 range need to be re-detected.
- 21.10 Samples with OD ratio failures must be re-detected.
- 21.11 Examples of unexpected results below:

ROW	Dilution Factor	Example 1	Example 2	Example 3	Example 4
A	1	2.610	2.564	0.812	3.126
B	5	2.461	2.684	0.0161	0.857
C	25	3.112	2.432	0.055	1.432
D	125	2.668	1.032	0.064	0.292
E	625	2.984	0.287	0.079	0.074
F	3125	1.568	0.074	0.052	0.066
Interpretation:		Very high titer specimen. Not an error.	High titer specimen. Not an error.	Low titer specimen. Not an error.	Error.

22. REPORTING RESULTS:

- 22.1 Interpretation of ACTG Results
 - 22.1.1 Each protocol has different cutoffs for high and low values decided by various protocol virologists. The results are loaded into a main spreadsheet then copied to site-specific spreadsheets for fax reporting.
 - 22.1.2 If this specimen is being done by the Boom method due to a previously missing qs and the results are the same with this method, comment in the computer or spreadsheet, “Unable to detect using Boom method.”

23. PROCEDURE NOTES:

- 23.1 Residual ethanol left on the pellet will inhibit the amplification.
- 23.2 Due to the high analytical sensitivity of this test and the potential for contamination, extreme care should be taken to preserve the purity of kit reagents or amplification mixtures.
- 23.3 All reagents should be closely monitored for purity. Discard any reagents that may be suspect.
- 23.4 Workflow in the laboratory should proceed in a uni-directional manner, beginning in the reagent preparation area, then the specimen preparation area, onto the Amplification/detection area.
- 23.5 Supplies should be dedicated to each activity and must not be used for other activities or moved between areas. Equipment and supplies used for reagent preparation/specimen preparation activities must not be used for pipetting or processing amplified DNA or other sources of target DNA.
- 23.6 Gloves must be worn in each and changed before leaving that area.
- 23.7 Good laboratory technique is essential to the proper performance of the assay.

24. LIMITATIONS OF PROCEDURE:

- 24.1 The presence of AmpErase in the AMPLICOR HIV-1 MONITOR Master Mix reduces the risk of amplicon contamination. However, contamination from HIV positive controls and HIV positive clinical specimens can be avoided only by good laboratory practices and careful adherence to the procedures specified above.
- 24.2 Use of this product should be limited to personnel trained in the techniques of PCR.
- 24.3 Only the Perkin-Elmer GeneAmp PCR System 9600 or GeneAmp 2400 thermal cyclers can be used with this product.
- 24.4 Monitoring the effects of antiretroviral therapy by serial measurements of plasma HIV-1 RNA has only been validated for patients with baselines viral loads \geq 25,000 copies/ml.
- 24.5 The performance of the Amplicor HIV-1 Monitor Test has only been validated with HIV subtype B specimens.
- 24.6 This test can accurately detect a 0.39 \log_{10} (2.8-fold) or greater change in HIV-1 RNA when the viral load is 50 – 75,000 copies/ml, a 0.44 \log_{10} (2.8-fold) or greater change when the viral load is approximately 75 copies/ml, and a 0.68 \log_{10} (5-fold) or greater change in HIV-1 RNA for patients whose viral load is approximately 50 copies/mL.

25. PERFORMANCE CHARACTERISTICS:

25.1 Reproducibility

25.1.1 Assay reproducibility was determined by assessing intra-assay, inter-assay and inter-lab variability. Reproducibility data were generated by testing 5 samples at three laboratories over a period of 10 days. The samples were prepared by dilution of viral stocks in HIV-negative human plasma and ranged in concentration from 75 to 50,000 copies/ml. The analysis of assay variability included separate assessments of intra-assay, inter-assay, inter-laboratory and total variance for specimens with HIV-1 RNA viral loads ≥ 75 copies/ml and for specimens with HIV-1 RNA viral loads of approximately 75 copies/ml and viral loads of approximately 50 copies/mL. The analyses were performed after the test results were \log_{10} transformed. The results from the specimens are shown in the following table:

RNA Level	# Labs	Variance Component	Standard Deviation	Variance	% of Total Variance
50 copies/mL	3	Total	0.2482	0.0616	100
		Inter-Lab	0.0316	0.0010	1.6
		Inter-Assay	0	0	0
		Intra-Assay	0.2462	0.0606	98.4
75 copies/mL	3	Total	0.2045	0.0422	100
		Inter-Lab	0.1288	0.0166	39.3
		Inter-Assay	0.0265	0.0007	1.7
		Intra-Assay	0.1579	0.0249	59.0
>75 copies/mL	3	Total	0.1661	0.0276	100
		Inter-Lab	0.0906	0.0082	29.8
		Inter-Assay	0.0814	0.0066	24.0
		Intra-Assay	0.1130	0.0128	46.2

25.2 To assess assay reproducibility at approximately 50 copies/mL, 75 copies/mL and ≥ 75 copies/mL, the standard deviation of the sum of the two important sources of assay variance (intra-assay and inter-assay) was determined and the 95% confidence interval for the standard deviation was calculated. See following table. These data show that the ultrasensitive procedure can accurately detect a $0.39 \log_{10}$ (2.8-fold) or greater change in HIV-1 RNA when the viral load is 75 – 75,000 copies/mL, a $0.44 \log_{10}$ (2.8-fold) or greater change when the viral load is approximately 75 copies/mL, and a $0.68 \log_{10}$ (5-fold) or greater change in HIV-1 RNA for patients whose viral load is approximately 50 copies/mL.

RNA Level	Σ of Variance*	SD of Variance	95% CI
50 copies/mL	0.0606	0.3481	$\pm 0.68 \log_{10}$
75 copies/mL	0.0256	0.2263	$\pm 0.44 \log_{10}$
> 75 copies/mL	0.0194	0.1970	$\pm 0.39 \log_{10}$

*for inter-assay and intra-assay variance

25.3 Specificity Studies

25.3.1 Analytical Specificity

25.3.1.1 The analytical specificity of this kit was evaluated by adding cultured cells, cultured virus or purified nucleic acid from the following organisms and viruses to HIV negative human plasma then analyzing these spiked samples. None of the non-HIV organisms, viruses or purified nucleic acids tested showed reactivity in the test. Three of the four HIV-2 isolates that were tested gave positive results. Only HIV-2 subtype A (isolate 7824A) was not detected by this kit; however no specific claims can be made for the ability of this test to amplify HIV-2 isolates.

Adenovirus type 2	Hepatitis B Virus	Human Papilloma virus 11
Adenovirus type 3	Hepatitis C Virus	Human papilloma virus 18
Adenovirus type 7	HIV-2BEN	HTLV I/II
Cytomegalovirus (AD-169)	HIV-2 subtype A/B (7312A)	Mycobacterium avium
Cytomegalovirus Davis	HIV-2 subtype A (60415K)	Propionibacterium acnes
Epstein-Barr virus P-3	HIV-2 subtype A (7824A)	Pneumocystis carini
Epstein-Barr virus HR1	Herpes simplex type I	Staphylococcus aureus
Epstein-Barr virus	Herpes simplex type II	Staphylococcus epidermidis

25.3.1.2 Limited testing has been performed on HIV-1 subtypes other than Subtype B. Preliminary experimental data in which synthetic nucleic acid constructs were used to represent the HIV-1 subtype mismatches with primers SK462 and SK431 indicate that non-B subtypes of HIV-1 will be amplified by the kit with reduced efficiencies. The reduced amplification efficiency depends upon the number of mismatches with the SK462 and SK431 primers. Group O specimens will not be amplified by this test.

25.3.2 Clinical Specificity

25.3.2.1 The clinical specificity of this test was determined by analyzing 495 anti-HIV-1-negative blood donors. None of these specimens was reactive with the kit. Assuming a zero prevalence of HIV-1 infection in the seronegative blood donors, the specificity of the test was 100%.

25.3.2.2 This test will amplify HIV-1 DNA if it is present in the processed specimen. Roche performed in-house testing using a modification of the Amplicor HIV-1 Monitor test which made the test specific and more sensitive for the qualitative detection of HIV-1 DNA. This modified kit was used on fifty-five HIV positive plasma specimens. This testing showed that fourteen specimens had plasma DNA present. Five of the fourteen specimens that were shown to contain plasma DNA were retested by the AMPLICOR HIV-1 Monitor Test but without the reverse transcription steps so that only plasma DNA would be

amplified. The results from these tests showed that DNA was undetectable by the AMPLICOR-1 HIV MONITOR Test in four of the specimens and contributed to less than 10% of the apparent HIV-1 RNA test result in the other specimen.

25.3.3 Sensitivity:

The analytical sensitivity of this kit was established by the determining the minimum number of copies of HIV-1 RNA that can be reproducibly detected by the test procedure. This was accomplished by analyzing serial dilutions of purified HIV RNA transcript. The results demonstrate that the AMPLICOR HIV-1 MONITOR Test could detect less than 2 copies of HIV RNA per reaction, and that 7.5 or more copies of HIV RNA per reaction were detected 100% of the time.

25.3.4 Limit of Detection

The lower limit of detection of the AMPLICOR HIV MONITOR test was determined by analysis of reconstructed HIV-positive plasma samples, prepared by serial dilution of well-characterized stocks of cultured HIV into HIV negative human plasma. Studies were conducted at three independent laboratories in the United States using two panels of 32 blinded specimens each, prepared using well characterized stocks of HIV spiked into HIV-negative human plasma. The concentration of viral RNA in the blinded specimens was determined prior to the study by virion associated HIV-1 RNA in plasma at concentrations as low as 50 RNA copies/mL plasma provided that the OD of the selected microwell is within the specified OD range (0.2 to 2.0).

25.4 Linear Range

Serial dilutions of two well characterized viral stocks were tested in replicates of six by three separate laboratories. The test results from the study sites were combined and plotted on a log-log scale. The ultrasensitive specimen processing was found to give a linear response between 50 and 75,000 HIV-1 RNA copies/mL.

26. CLINICAL APPLICATION AND INTERPRETATION:

26.1 Application

26.1.1 This test has been used as an aid in assessing viral response to antiretroviral treatment as measured by changes in plasma HIV-1 RNA levels. The clinical significance of changes in HIV RNA measurements has not been fully established although several large studies that will more fully determine the role of comparative HIV RNA measurements in patient management are now in progress. HIV-1 RNA levels as measured by PCR were used as one of the surrogate markers in the accelerated approval process for the protease inhibitor drugs INVIRASE, CRIVAN, and NORVIR, and for the reverse transcriptase inhibitor drug

EPVIR. The utility of plasma HIV-1 RNA in surrogate endpoint determinations has not been fully established.

- 26.1.2 Within three to six weeks of exposure to HIV, infected individuals generally develop a brief, acute syndrome characterized by flu-like symptoms and associated with high levels of viremia in the peripheral blood. In most infected individuals this followed by an HIV-specific immune response and a decline of plasma viremia, usually within four to six weeks of the onset of symptoms. After seroconversion infected individuals typically enter a clinically stable, asymptomatic phase that can last for years. The asymptomatic period is characterized by persistent, low level plasma viremia and a gradual depletion of CD4+ T lymphocytes, leading to severe immunodeficiency, multiple opportunistic infections, malignancies and death. Although virus levels in the peripheral blood are relatively low during the asymptomatic phase of the infection, virus replication and clearance appear to be dynamic processes in which high rates of virus production and infection of CD4+ cells are balanced by equally high rates of virus clearance, death of infected cells and replenishment of CD4+ cells, resulting in relatively stable levels of both plasma viremia and CD4+ cells.
- 26.1.3 Quantitative measurements of HIV viremia in the peripheral blood have shown that higher virus levels may be correlated with increased risk of clinical progression of HIV disease and that reductions in plasma virus levels may be associated with decreased risk of clinical progression. Virus levels in the peripheral blood can be quantitated by measurement of the HIV p24 antigen in serum, by quantitative culture of HIV from plasma, or by direct measurement of viral RNA in plasma using nucleic acid amplification or signal amplification technologies.
- 26.1.4 P24 antigen is the principle core protein of HIV and is found in serum either free or bound by anti-p24 antibody. Free p24 antigen can be measured with commercially available enzyme immunoassays (EIA), although the usefulness of p24 antigen as a marker of viral load is limited since the antigen is detectable in only 20% of asymptomatic patients and 40-50% of symptomatic patients. Procedures to dissociate antigen-antibody complexes improve the sensitivity of the p24 antigen tests, but the viral protein remains undetectable in most asymptomatic patients.
- 26.1.5 Infectious HIV in plasma can be cultured by inoculation into activated peripheral blood mononuclear cells (PBMC) from normal donors. Quantitation is achieved by inoculating PBMC with serial dilutions of the plasma specimen. Quantitative culture has limited utility for monitoring virus levels in levels in infected individuals since only a small fraction of virus particles is infectious in vitro. Infectious virus is often undetectable in asymptomatic patients.
- 26.1.6 HIV RNA in plasma can be quantitated by nucleic acid amplification technologies, such as PCR. The AMPLICOR HIV-1 MONITOR test uses PCR technology to achieve maximum sensitivity and dynamic range for quantitative detection of HIV-1 RNA in plasma.

26.2 Clinical Performance

26.2.1 This use of the AMPLICOR HIV-1 MONITOR test to predict the risk of disease progression in HIV infected individuals was evaluated in ACTG studies 116A and 116B/117. The data from these studies were analyzed by the Cox Proportional Hazards Model to evaluate the frequency of disease progression based upon HIV-1 RNA level. ACT 116A was a double blinded study that compared the clinical efficacy of zidovudine (ZDV) in combination with two doses of 2',3' -dideoxyinosine (ddl) in patients with advanced HIV disease who had up to 16 weeks of prior treatment with zidovudine. ACTG Study 116B/117 was an efficacy study which compared ddl and ZDV therapy of patients with HIV infection who had been on ZDV treatment for more than 16 weeks. The patient population in each of these studies included patients with a diagnosis of AIDS at study baseline, ARC at baseline, and asymptomatic patients at baseline. Disease progression was defined as progression to AIDS, new AIDS defining event, or death.

26.3 The unadjusted and adjusted relative hazards for disease progression as measured by baseline HIV-1 RNA levels, change in HIV-1 RNA levels over 8 weeks, and CD4+ cell counts were evaluated using Ox Proportional Hazards Models. The unadjusted Relative Hazard represents the risk conferred by the variable for other variables in the model. These models give the increased risk (if any) of disease progression associated with the variables entered into the model. The analyses were performed by assessing the relative hazards of a 5-fold difference in the study variable. These data show that in population of patients with advanced HIV disease and undergoing specific anti-reverse transcriptase therapies, 5-fold higher baseline HIV-1 RNA levels are associated with increased risk of disease progression. For patients who have had greater than 16 weeks prior ZDV therapy, 5-fold higher baseline HIV-1 RNA levels have not proven to be of significant prognostic value. For patients who have had either no prior ZDV therapy or up to 16 weeks or less of ZDV therapy, 5-fold changes between baseline and week 8 RNA levels have significant prognostic value. For patients who have had greater than 16 weeks of prior ZDV therapy, 5-fold changes between baseline and week 8 RNA levels have not proven to be of significant prognostic value.

26.4 The frequency of disease progression was also analyzed for each study by dividing each study population into deciles by rank order of baseline HIV-1 RNA. The deciles were evaluated for the frequency of disease progression. For each study, a frequency of disease progression of $\geq 60\%$ was found for all patients with baseline HIV-1 RNA levels above 250,000 copies/mL. In study 116a, an approximate 35% frequency of disease progression was found for patients in the first four deciles (<11912, <34661, <72438, and <103806 HIV-1 RNA copies/mL). The frequency of disease progression was between 40% and 50% in the next three deciles (<15695, <194312, and <247229). In the last three deciles, where the HIV-1 RNA levels were >250,000 HIV-1 RNA copies/mL, the

frequency of disease progression was greater than 60%. In study 116B/117 the frequency of disease progression was found to be more variable, but still showed the general trend of higher rates of progression with increased HIV-1 RNA levels. An average 30% disease progression rate (range 10% - 60%) was found for the first six deciles in this study (<11571, <31292, <49743, <62132, <97881, and <150866). Their rate of disease progression increased to 50% and 60% for the next two deciles (<251327 and <403146). The disease progression rates were greater than 80% for the last two deciles (<794027 and <1456302) where the HIV-1 RNA levels were greater than 7030000.

- 26.5 The use of the AMPLICOR HIV-1 MONITOR test to measure the effects of antiretroviral therapy was evaluated in two separate clinical studies of antiretroviral compounds including reverse transcriptase inhibitors (nucleoside analogues) the protease inhibitor drug Saquinavir (Inverase) and combinations of these two classes of drugs. These two studies were ACTG 229 and NV 14256. ACTG 229 was a double blind phase II randomized study whose primary objective was to evaluate immunologic activity (CD4 counts), the reduction in HIV viral load (PBMC quantitative cultures), and the safety and tolerability of three different treatment arms: ZDV in combination with ddC, Inverase in combination with ZDV, and Inverase in combination with ZDV and ddC. Study NV 14256 was a double blind phase III randomized study whose primary objective was to evaluate the safety, tolerability and efficacy of the three treatments (ddC, Inverase, Inverase and ddC) based on clinical endpoints in patients discontinuing or unable to take ZDV, and to compare survival among the three treatment groups. In both studies prior ZDV treatment was an inclusion criterion. IN ACTG 229 the patients had almost two years of ZDV treatment, and in study NV 14256 the majority had over one year of prior ZDV treatment. The demographic and baseline disease characteristics of the patients in these studies represented a diverse population of patients with advanced HIV-1 infection and a wide range of prior antiretroviral treatment.
- 26.6 The use of the AMPLICOR HIV-1 MONITOR test to measure the effects of treatment over time was evaluated by analyzing the median change from baseline and the DAVG (difference averaged over time-mean change from baseline over weeks). To evaluate the ability of the AMPLICOR HIV-1 MONITOR test to detect changes in HIV-1 RNA levels as a result of therapy, the medians of change from baseline were analyzed over time for each treatment arm of each study. The DAVG were analyzed for each treatment arm of each study with medians, upper and lower quartiles estimated. The correlation of the DAVG for the AMPLICOR HIV-1 MONITOR test versus quantitative PBMC microculture was evaluated as was the correlation of the DAVG for the AMPLICOR HIV-1 MONITOR test versus CD4+ cell count.
- 26.7 Measurable and sustained decreases in HIV-1 RNA levels as determined by the AMPLICOR HIV-1MONITOR test were seen. In each study the largest and the most sustained median changes in HIV-1 RNA levels at each time point were seen in patients in the combination treatment arms

(reverse transcriptase inhibitors/protease inhibitors). These studies have not yet correlated changes in viral RNA levels with changes in viral resistance or changes in disease progression.

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Procedure: ACTG Lab Man UltraSensitive Roche Monitor Test, v1.5- Boom Extraction

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