UltraSensitive Roche Monitor Test, v1.5- MWP

1. PRINCIPLE

1.1 Name and Intended Use

1.1.1 The AMPLICOR HIV-1 MONITOR test version 1.5 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 can quantitate HIV-1 RNA over the range of 50-100,000 copies/mL. The test is intended of use in conjunction with clinical presentation and other laboratory markers as an indicator of disease prognosis of HIV-1 infected patients, and for monitoring the effects of anti-retroviral therapy by serial measurements of plasma HIV-1 RNA for patients with baseline viral loads equal to or greater than 25,000 copies of HIV-1 viral RNA/mL. This test is not to be used as a screening test for blood or blood products 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. 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 is 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 higher 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.

1.2.2 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 HIVp24 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.

1.2.3 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.

1.2.4 P24 antigen is the principle core protein of HIV and is found in serum either free or bound by anti-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 antigens detected 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 symptomatic patients.

1.2.5 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 specimens. Quantitative culture has limited utility for monitoring virus levels in infected individuals since only a small fraction of virus particles is infectious in vitro. Infectious virus is often undetectable in asymptomatic individuals.

1.2.6 HIV RNA in plasma can be quantitated by nucleic acid amplification technologies such as PCR. This test utilizes PCR technology to achieve maximum sensitivity and dynamic range for the quantitative detection of HIV-1 RNA in EDTA or ACD anti-coagulated plasma.

1.2.7 PCR allows the exponential amplification of a nucleic acid sequence. 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 and has been developed to yield equivalent quantification of group M subtypes of HIV-1. The performance characteristics of the test with Group O specimens have not been determined.

1.3.3 The quantitation of HIV-1 viral RNA is performed using a 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.

1.4 SPECIMEN PREPARATION

1.4.1 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.

1.5 REVERSE TRANSCRIPTION AND PCR AMPLIFICATION

1.5.1 *Target Selection*

Selection of the target RNA sequence for HIV-1 depends on identification of regions within the HIV-1 genome that show maximum sequence conservation. Accordingly, the appropriate selection of primers and probe is critical to the ability of the test to detect the HIV-1 genotype. The AMPLICOR HIV-1 MONITOR test v1.5 uses the primers SK145 and SKCC1B to define a sequence of 155 nucleotides within the highly conserved region of the HIV-1 *gag* gene. The *gag* region encodes the group specific antigens or core structural proteins of the virion. The HIV-1 *gag* genes are generally about 1500 nucleotides in length and are located at the appropriate positions 789-2290 in the HIV genome. The nucleotide sequence of the primers has been optimized to yield equivalent amplification of Group M subtypes of HIV-1.

1.5.2 *Reverse Transcription*

The reverse transcriptase and PCR amplification reactions are performed with the thermo stable recombinant enzyme *Thermus thermophilus* DNA polymerase (r*Tth* pol). In the presence of manganese and under the appropriate buffer conditions, r*Tth* pol has both reverse transcriptase and DNA polymerase activity. This allows both reverse transcription and PCR amplification to occur in the same reaction mixture.

1.5.3 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 (SKCC1B) and the upstream or sense primer (SK145) are biotinylated at the 5' ends. The reaction mixture is heated to allow the downstream primer to anneal specifically to the HIV-1 target RNA and to the HIV-1 QS target RNA. In the presence of Mn^{2+} and excess deoxynucleoside triphosphates (dNTPs), including deoxyadenosine, deoxyguanosine, deoxycytidine, deoxyuridine and deoxythymidine triphosphates, r*Tth* pol extends the annealed primer forming a (cDNA) DNA strand complementary to the RNA target.

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'-tetramethylbenxidine (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.

1.8 HIV-1 RNA QUANTITATION

1.8.1 The AMPLICOR HIV-1 RNA MONITOR Test v1.5 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 233 nucleotide in vitro transcribed RNA molecule with primer binding region identical to those of the HIV-1 target sequence. The QS, therefore, contains SK145 and SKCC1B primer binding sites and generates a product of the same length (155 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.

1.8.2 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:

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

2.0 SPECIMEN:

2.1 Type:

2.1.1 This test is licensed for use with plasma specimens only. Other specimen types may be assayed for research purposes only and may require special processing to remove inhibitory substances. The anticoagulants EDTA and ACD have been evaluated and been found to be acceptable. The use of ACD anticoagulated specimens will yield results that are approximately 15% lower than test results obtained from EDTA anticoagulated specimens due to the dilution effect of the 1.5ml of ACD anticoagulant present in the blood collection tube. The minimum volume is a 2ml tube yielding 2 aliquots of 250 to 500 □l of plasma each. The ideal volume is a 7ml tube yielding 2 aliquots of 1ml each.

2.1.2 Serum specimens and specimens collected in **heparin** are unsuitable for this test. If necessary, these specimens may be assayed following a boom extraction procedure.

2.2 Standard (universal) precautions will be observed for the collection, handling, transport and processing of <u>all</u> specimens.

2.3 **Handling Conditions:**

2.3.1 Separate plasma from whole blood within 6 hours of collection by centrifugation at 800 -1600 x g for 20 minutes at room temperature. Alternatively, spin at 800 x g for 10 minutes. Remove the plasma to a clean centrifuge tube. The resulting plasma should then be spun at 800 x g for an additional 10 minutes for clarification.

2.3.2 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.

3. MATERIALS AND EQUIPMENT:

3.1 **Materials: AMPLICOR HIV-1 MONITOR test kit, v 1.5**

3.1.1 Specimen Preparation Reagents
- AMPLICOR HIV-1 MONITOR Lysis Reagent
- AMPLICOR HIV-1 MONITOR Quantitation Standard
- AMPLICOR HIV-1 MONITOR Specimen Diluent
- Isopropanol
- 95% Ethanol reagent grade for Molecular Biology or Histology (**not denatured**), 70% v/v with deionized water

3.1.2 Control Reagents
- Negative Plasma (NHP)
- AMPLICOR HIV-1 MONITOR Negative Control (HIV-1 (-)C)
- AMPLICOR HIV-1 MONITOR Low Positive Control (HIV-1 L(+)C)
- AMPLICOR HIV-1 MONITOR High Positive Control (HIV-1 H(+)C)

3.1.3 VQA Controls

3.1.4 Amplification Reagents
- AMPLICOR HIV-1 MONITOR Master Mix
- AMPLICOR HIV-1 MONITOR Manganese Solution

3.1.5 Detection Reagents
- MONITOR Denaturation Solution
- MONITOR Hybridazation Buffer
- AMPLICOR Avidin-HRP Conjugate
- AMPLICOR Substrate A
- AMPLICOR Substrate B
- AMPLICOR Stop Reagent
- AMPLICOR 10X Wash Concentrate
- Distilled or deionized water
- AMPLICOR HIV-1 MONITOR Microwell Plate

3.2 Equipment:

3.2.1 Applied Biosystems (Perkin Elmer) GeneAmp® PCR system 9600 or 2400 thermal cycler

3.2.2 Consumables: tubes, caps, base, tray, and retainer

3.2.3 Aerosol resistant pipette tips capable of holding 50 to 1000 microliters

3.2.4 Pipettors, adjustable volume (20 – 200 μl), (50 μl), (200 μl) Pipettes should be within 3% of stated volume.

3.2.5 Impact Pipettor

3.2.6 Narrow tip, sterile transfer pipettes
3.2.7 Nitrile gloves, **powder-free**
3.2.8 Cryovials with caps, sterile, 2ml capacity
3.2.9 Microwell plate sealers
3.2.10 isposable reagent reservoirs
3.2.11 Disposable plastic bags
3.2.12 Biological Safety Cabinet (BSC), Template Tamer Box, or equivalent
3.2.13 Microwell plate Washer capable of washing a 96-well plate at 30 second intervals
3.2.14 Microwell plate 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
3.2.15 Centrifuge
3.2.16 Microcentrifuge max RCF 16,000 x g, min RCF 12,500 x g
3.2.17 Refrigerated ultracentrifuge and fixed angle rotor (45 degrees capacity of 24 1.5mL tubes) with a RCF of 23,600 x g Rotor lid must form aerosol-tight seal to rotor to contain aerosols in case of tube failure
3.2.18 Dry incubator 37°C(± 2°C)
3.2.19 Personal computer with spreadsheet software or LDMS software
3.2.20 Graduated cylinders, 100 to 1000 microliter capacities
3.2.21 Sterile bottles
3.2.22 Disposable pipettes, 5ml and 10ml
3.2.23 Absorbent backed paper
3.2.24 Disposable gown
3.2.24 Hypochloride or equivalent
3.2.25 Biohazard disposal system
3.2.26 Vortex mixer

3.3 WARNINGS AND PRECAUTIONS:

3.3.1 For in Vitro diagnostic use.
3.3.2 This test is for use with human plasma collected in EDTA or ACD anticoagulants only. Heparin has been shown to inhibit PCR and must not be used with this procedure.
3.3.3 Do not pipette by mouth.
3.3.4 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.
3.3.5 Avoid microbial and ribonuclease contamination of reagents when removing aliquots from reagent bottles. The use of sterile disposable pipettes and barrier pipette tips are recommended.
3.3.6 Do not pool reagents from different lots or from different bottles of the same lot.
3.3.7 Dispose of unused reagents and waste in accordance with country, federal, state and local regulations.
3.3.8 Do not use kit after expiration date.
3.3.9 Material Safety Data Sheets (MSDS) are available on request from Roche Response Center at 1-800-428-5030.

3.3.10 orkflow 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, 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.

3.3.11 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.

3.3.12 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.

3.3.13 HIV-1 QS, HIV-1 DIL, HIV-1 MMX, HIV-1 Mn^{2+}, 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.

3.3.14 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.

3.3.15 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.

3.3.16 Do not allow HIV-1 LYS, which contains guanidine thiocynate, to contact sodium hypochlorite solution. This mixture can produce a highly toxic gas.

3.3.17 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.

3.4 PREPARATION:

3.4.1 Specimen Preparation Reagents:

3.4.1.1 AMPLICOR HIV-1 MONITOR Lysis Reagent

A tris buffered solution containing guanidine thiocyanate, dithiothreitol and glycogen.
Store at 2 to 8°C until expiration date. If a precipitate forms during storage, warm to ambient temperature and mix thoroughly prior to use.Warm for a maximum of 30 minutes. Examine each bottle prior to use against a white background for appearance of yellow color or signs of leakage. If there is any yellow color or evidence of leakage, do not use the bottle. Contact Roche for replacement. Once opened, any unused portion must be discarded.

3.4.1.2 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. Once opened, any unused portion must be discarded.

3.4.1.3 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.

3.4.1.4 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. Once opened, any unused portion must be discarded.

3.4.1.4 Isopropanol(2,2 – propanol)
Store at room temperature in the flammable liquids cabinet.

3.4.1.5 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 ethanol in flammable liquids cabinet. Prepare fresh 70% daily.

3.4.2 Control Reagents:

3.4.2.1 VQA Controls
A solution containing positive control supernatant spiked into seronegative plasma at varying concentrations. Store at –70°C until expiration date.

3.4.2.2 Negative Plasma (NHP)
Human plasma that has been found to be non-reactive by FDA licensed test for antibody to HCV, antibody to HIV-1/2, and HbsAg, containing ProClin 300. Store at 2 to 8°C until expiration date.

3.4.2.3 AMPLICOR HIV-1 MONITOR Negative Control (HIV-1 (-)C)
A buffered solution containing poly rA RNA, Tris-HCL, EDTA, and 0.05% sodium azide.
Store at 2 to 8°C until expiration date.

3.4.2.4 AMPLICOR HIV-1 MONITOR Low Positive Control (HIV-1 L(+)C)
A buffered solution containing a non-infectious RNA transcript with HIV-1 sequence, poly rA RNA, Tris-HCl, EDTA, and 0.05% sodium azide. Store at 2 to 8°C until expiration date.

3.4.2.5 AMPLICOR HIV-1 MONITOR High Positive Control (HIV-1 H(+)C)

A buffered solution containing a non-infectious RNA transcript with HIV-1 sequence, poly rA RNA, Tris-HCl, EDTA, and 0.05% sodium azide. Store at 2 to 8°C until expiration date.
Once opened, any unused portion must be discarded.

3.4.3 **Amplification Reagents**

3.4.3.1AMPLICOR HIV-1 MONITOR Master Mix
A bicine buffered solution containing <37% glycerol, potassium acetate, <0.07% dATP, dCTP, TTP, dUTP, <0.001% biotinylated primers, <0.01%r *Tth* Pol, <0.01% AmpErase and 0.05% sodium azide.
Store at 2 to 8°C until expiration date.

3.4.3.2AMPLICOR HIV-1 MONITOR Manganese Solution
A solution containing <2% manganese acetate, amaranth dye and 0.05% sodium azide. Store at 2 to 8°C until expiration date.

3.4.3.3Working 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.

3.4.4 Detection Reagents:

3.4.4.1MONITOR Denaturation Solution
A solution of EDTA, 1.6% sodium hydroxide, and amaranth dye.
Store at 2 to 25°C until expiration date. Once opened, any unused portion must be discarded.

3.4.4.2MONITOR Hybridization Buffer
A sodium phosphate solution containing <0.2% solubilizer and <25% sodium thiocyanate.
Store at 2 to 25°C until expiration date. Once opened, any unused portion must be discarded.

3.4.4.3AMPLICOR Avidin-HRP Conjugate
An avidin-horseradish peroxidase conjugate in a buffered solution containing 1% ProClin 150™, emulsifier Emulsit 25, 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.

3.4.4.4AMPLICOR Substrate A
A citrate solution containing 0.01% H_2O_2 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.

3.4.4.5AMPLICOR 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.

3.4.4.6Working Substrate Solution

For each microwell plate, 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.

3.4.4.7AMPLICOR Stop Reagent

Contains 4.9% sulfuric acid.

Store at 2 to 25°C until expiration date Once opened, any unused portion must be discarded..

3.4.4.8AMPLICOR 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.

3.4.4.9Wash Buffer 1X

Dissolve any precipitate by warming to 30 to 37°C. Add 100ml of 10X wash concentrate to 900ml of distilled, deionized water. Mix well. Store in a clean, closed container at 2 to 25°C for up to 2 weeks. Label with preparation and expiration dates.

3.4.4.10AMPLICOR HIV-1 MONITOR Microwell Plate

An oligonulceotide 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 stored in the resealable pouch.

Storage of kits: Do not freeze reagents.

4.0 CALIBRATION:

None

5.0 QUALITY CONTROL:

5.1 Three kit controls as well as 1.5 $\log_{10}$ 3 must be included with every run[1]

- 5.1.1 A run contains a maximum of four plates[2]
- 5.1.2 A minimum of one control must be included on each plate within a run.
- 5.1.3 When a fourth plate run is performed, the fourth plate will include at a minimum, one external VQA control (1.5 $\log_{10}$ 3).
- 5.1.4 It is recommended that a full set of VQA controls must be run for each new kit lot implemented in the lab to identify potential shifts in kit performance

5.2 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.

5.3 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.

5.4 Basic Criteria to define a valid run for the Roche HIV-1 Monitor™ Test

5.4.1 All kit copy control results must be valid
5.4.1.1 No out of sequence errors in the 0.2-2.0 working OD range
5.4.1.2 QS result must be valid- OD must be between 0.3-2.0 in 1:1 or 1:5 dilution
5.4.1.3 No OD ratio failures (run may be considered valid after re-detection of control)
5.4.1.4 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.
5.4.2 All Kit positive control results must be within the range provided by the manufacturer
5.4.2.1 if outside range, all samples within the run need to be repeated
5.4.2.2 control can be re-detected in cases where applicable
5.4.3 Kit negative control must be undetectable- all WT ODs <0.2
5.4.4 The VQA will provide 3 standard deviation ranges for each lot of VQA controls

Table 1: Theoretical Range for Demonstrative Purposes[9]

VQA CC	Assay	Assay SD Log_{10} 10 units[10]	-3SD	-2SD	-1SD	Median	+1SD	+2SD	+3SD
1.5 log_{10} 3	**UltraSensitive**	**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.

5.5 The VQA panel of 1.5 log_{10} 0, 2, 3 and 4 should be assayed to validate new kit lots or shipments.

5.6 Kit Lot Entry in the LDMS system
Log in Roche kit information into the QA/QC Kit Entry module. This includes kit lot, QS input, kit controls and ranges. Separate entry is required for each assay used- e.g. Standard and UltraSensitive.
Log in VQA control information into the QA/QC VQA Entry module, including lot and ranges (VQA control information sheets may be retrieved from the website at

http://aactg.s-3.com/vqa.htm). Separate entry is required for each assay used- e.g. Standard and UltraSensitive

6.0 TROUBLESHOOTING/MAINTENANCE:

6.1 Maintenance is to be performed on the plate washer, reader, and thermal cycler per the manufacturer. To troubleshoot the thermal cycler, check the Applied Biosystems/Perkin Elmer handbook.

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

7.0 PROCEDURE-STEPWISE:

Preliminary Statements

7.1 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.

7.2 Plasma samples must be at ambient temperature before use.

7.3 Screw cap tubes must be used for specimen and control preparation to prevent splashing and potential cross contamination of specimens and controls.

7.4 Pre-cool the ultracentrifuge and rotor to 2 – 8 °C as described in the operating manual for the ultracentrifuge.

7.5 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 are 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.

7.6 Run Size: Each kit contains sufficient reagents for 2 -12 test batches, which may be performed separately or simultaneously. It is recommended that one replicate of the HIV-1 Monitor (-) control, (L+) control, (H+) control, and 1.5 $\log_{10}3$ be included in each run. If three plates are assayed, then there will be a control on each plate with 2 controls on the first plate. If two plates are assayed, then there will be two controls on the first plate and the two controls will be on the third plate. In the event that four plates are assayed, there will be a control on each plate.

7.7 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.

7.8 **Reagent Preparation**

7.8.1 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.

7.8.2 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.

7.8.3 Pipette 50 microliters of working master mix into each PCR tube using a micropipettor with an aerosol resistant tip. Discard leftover working master mix.

7.8.4 Place the microtube tray in a plastic zip-lock bag and store the tray at 2-8°C until the specimen preparation is completed. **Amplification must begin within 4 hours of the preparation of the working master mix**.

7.8.5 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. Remove gloves and dispose of them properly. Remove lab coat and/or any other protective garments.

7.9 **Specimen and Control Preparation**

7.9.1 Prepare 70% ethanol, volumes depend on if using 95% ethanol. For 12 t ests, mix 11 ml of 95% ethanol and 4ml distilled water.

7.9.2 Prepare working Lysis Reagent as follows:
Warm the cold Lysis Reagent until all crystallization is gone from the reagent. Do not over warm.
Mix for at least ten seconds prior to use to ensure that it is thoroughly mixed.
For each batch of 12 samples, add 25ul of Quantitation Standard (QS) to one bottle of the Lysis Reagent. Mix well.
The pink dye in the QS is for visual confirmation that it has been added to the Lysis Reagent.
Discard any leftover working Lysis Reagent after completion of the specimen extraction procedure. Working reagent is good for 4 hours.

7.9.3 Label a 1.5 ml screw cap microcentrifuge tube for each sample, including one tube for each kit control and/or standard.

7.9.4 Thaw plasma specimens and standards to room temperature. Vortex each tube for 3-5 seconds.

7.9.5 Briefly centrifuge each specimen tube in order to collect the sample into the base of the tube. Do not contaminate gloves while manipulating specimens.

7.9.6 Add 500ul of each patient and log 3 to the appropriately labeled tube.

7.9.7 Add 500ul of NHP to each of the appropriately labeled control tubes.

7.9.8 Put an orientation mark on each tube and place the tubes into the ultracentrifuge with the orientation marks facing outward, so that the pellet will align with the orientation mark. Centrifuge at 23,000 x g for 60 minutes at 2-8'C.

7.9.9 Using a new fine tip disposable transfer pipette for each tube, carefully remove and discard the supernatant from each tube, being careful no to disrupt the pellet. Remove as much liquid as possible without disturbing the pellet. The pellet may not be visible. Withdraw the supernatant slowly allowing the liquid to drain completely off the sides of the tube. Do not use vacuum aspiration. Virus pellets obtained are stable for up to 6 hours at room temperature and at least 14 days at –20'C or colder

7.9.10 Dispense 600µl of working Lysis Reagent into each labeled microcentrifuge tube. Check that the working lysis reagent is pink to confirm that the QS was added to the lysis reagent.

7.9.11 Vortex the kit controls for at least 3-5 seconds. Add 12.5ul of the control to the corresponding tube containing working lysis reagent. Cap and vortex for at least 3-5 seconds.

7.9.12 Incubate the tubes for 10 minutes at room temperature.

7.9.13 Remove the cap from each tube and add 600µl of 100% Isopropanol to each tube. Re-cap the tubes and vigorously vortex them for 3-5 seconds.

7.9.14 Place the tubes into the microcentrifuge with the tube labels facing inward, so that the pellet will align with the opposite side of the tube. Centrifuge the tubes at maximum speed (at least 12,500 x g) for 15 minutes at room temperature. Upon completion of the centrifugation carefully remove each tube from the rotor, **carefully marking on the outside of the tube with a fine tip marker the position of the pellet (whether visible or not)**.

7.9.15 Beginning with the control tubes or the standard tubes, carefully draw off the supernatant, without disturbing the pellet (which may not be visible), using a fine tip, disposable transfer pipette. **Remove as much liquid as possible without disturbing the pellet: slide the pipette down the inside of the tube along the side opposite the pellet while drawing off the liquid. Maintain a continuous negative pressure with the transfer pipette as you draw off the liquid**.

7.9.16 Add 1ml of 70% ethanol to each tube, re-cap, and vigorously vortex 3-5 seconds.

7.9.17 Place the tubes into the microcentrifuge with the label facing inward so that the pellet will align with the opposite side of the tube, and centrifuge the sample at maximum speed for 5 minutes at room temperature.

7.9.18 Carefully remove the tubes from the centrifuge rotor and aspirate the supernatant as described as above. The pellet should be clearly visible at this time. **Remove as much of the supernatant as possible! (Do this twice). Residual ethanol can inhibit the amplification**.

7.9.19 Add 100µl of Specimen Diluent, re-cap, and vigorously vortex for at least 10 seconds to resuspend the extracted RNA. Note that insoluble material often remains.

7.9.20 Amplify the processed specimens within 2 hours of preparation or store frozen at -20°C for up to one week.

7.9.21 Preparation for amplification: If previously frozen, warm extracted specimens to room temperature, vigorously vortex each tube for at least 10 seconds.

7.9.22 Pipette 50µl of extracted specimens, controls or, if used, standards to the appropriately designated reaction tubes which were previously prepared using a micropipettor with a plugged tip. Use a new tip for each specimen and control.

7.9.23 Securely cap each tube and using the MicroAmp capping tool seal the tubes.

7.9.24 Transfer the tray with sealed tubes containing the processed specimens and controls in working master mix to the Amplification/Detection area.

7.9.25 Decontaminate work area with a 1:10 dilution of bleach. Follow by cleaning area with propanol.

7.9.26 Amplification must be started within 45 minutes of the time that the processed specimens and controls are added to the reaction tubes containing Working Master mix. The remainder of the processed specimens may be frozen and stored at –20'C or colder for up to one week with no more than one freeze thaw.

7.10 Reverse Transcription and Amplification

NOTE: Turn on the GeneAmp PCR System 9600 or 2400 thermal cycler at least 30 minutes prior to beginning amplification.

7.10.1 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**.

7.10.2 Make certain that the cover knob is turned completely counterclockwise and slide the cover forward.

7.10.3 **Turn the cover knob clockwise until hand tight**.

7.10.4 Program the GeneAmp System 9600 thermal cycler as follows:

7.10.5	Hold	2 minutes at 50°C
7.10.6	Hold	30 minutes at 60°C
7.10.7	78 cycles	10 seconds at 95°C, 10 seconds at 52°C, 10 seconds at 72°C
7.10.8	23 cycles	10 seconds at 90°C, 10 seconds at 55°C, 10 seconds at 72°C
7.10.9	Hold	15 minutes at 72°C

7.10.10 On the CYCLE programs the ramp time and allowed set point error s should be left at the default settings of 0:00, which is the maximum rate and 2'C respectively. Link the 5 programs together into a METHOD program.

7.10.11 Start the method program. The program runs for approximately one hour and 30 minutes.

7.10.12 Remove the reaction tray from the thermal cycler before 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**.

7.10.13 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).

7.10.14 The detection amplicon can be held at room temperature no more than 2 hours before proceeding to the detection reaction. If the detection reaction cannot be performed within this time, re-cap the tubes and store the denatured amplicons at 2 to 8°C for up to one week.

7.11 Detection

7.11.1 Warm all reagents and amplicons to room temperature prior to proceeding with the detection.

7.11.2 Prepare a sufficient amount of working Wash Solution (dilute 1 part of the 10 X solution with 9 parts of distilled or deionized water). This working Wash Solution is stable for up to 2 weeks at room temperature. Label with preparation and expiration date

7.11.3 Allow the microwell detection plate to warm to room temperature before removing it from its foil pouch.

7.11.4 Add 100µl of MONITOR Hybridization Buffer to each well using Program 2 on the IMPACT Pipettor.

7.11.5 Add 25µl 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 25µl from row A to B and mix as before. Continue through row F. Mix row F as before, then remove and discard 25µl. Discard pipette tips. This may be done using the IMPACT Pipettor Program 3 two times, where 25µl is transferred and mixes by pipetting 60µl up and down 10 times, and aspirates 25µl.

7.11.6 Add 25µl 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 25µl from row G to row H, again, mix as described in step5; remove and discard 25µl from row H along with the pipette tips.

7.11.7 Cover the plate and incubate it for 1 hour at 37°C (± 2°C).

7.11.8 Wash the entire detection plate 5 times with the working Wash Solution and an automated microwell plate washer. The microwell plate washer should entirely fill each well (400-450µl), allow each row (or column) soak for 30 seconds, then aspirate the entire contents of each well before proceeding to the next cycle.

7.11.9 Add 100µl of Avidin-HRP conjugate to each well, cover plate and incubate for 15 minutes at 37°C (±2°C).

7.11.10 Wash the plate as described in step 8.

7.11.11 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.

7.11.12 Pipette 100µl 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.

7.11.13 Add 100µl of Stop Reagent to each well (blue color will turn to a yellow color).

7.11.14 Measure the optical density at 450 angstroms within 10 minutes of adding the Stop Reagent. If this time is extended, less dilute wells will precipitate resulting in a lower OD.

7.11.15 Decontaminate work area with a 1:10 bleach solution.

8.0 CALCULATIONS:

8.1 Manual Calculations

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

8.1.1.1 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.

8.1.1.2 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).

8.1.1.2 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).

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

8.1.3 Calculate the “total HIV OD” by multiplying the value derived in step 2 by the dilution factor associated with that well.

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

8.1.4.1 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.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.

8.1.4.2 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 nto decrease from well G to well H).

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

8.1.6 Calculate the “total QS OD” by multiplying the value derived in step 5 by the dilution factor associated with that well.

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

HIV-1 RNA Copies/mL Plasma = (“total HIV OD”/”total QS OD”) X Input QS Copies per reaction X 40.

8.1.8 The dilution ratio must be checked for dilution errors. Follow instructions provided by the VQA.

8.2 LDMS Calculations

8.2.1 Follow the instructions for LDMS use in the LDMS manual.

8.2.2 Ultrasensitive Roche. Use Roche Ultrasensitive (Kit Controls) template.
8.2.3 Kit controls and the VQA 1.5 $\log_{10}3$ control are pre-loaded. You cannot modify/delete controls on templates.
8.2.4 The LDMS will automatically re-distribute the controls as specimens are added past plate one.
8.2.5 Invalid controls may be re-detected
8.2.6 On LDMS System, go to the assay module.
8.2.7 Click on the (+) sign next to Viral Load RNA from the Assay lists box.
8.2.8 Highlight the Roche Ultrasensitive (Kit Control) assay.
8.2.9 Then click on the New Run/Not Setup button on upper right side of Search Criteria box.
8.2.10 Click on the Select button at the bottom of the Assay Selection screen. The Filters/Criteria screen appears. The best suggestion at present to find specimens is to select the group from the Group combo box. Then narrow your search, create query statements using the Field, Operator and Value combo boxes.
8.2.11 Click the Find Specimens button at the bottom of the screen. This will take you to the Specimens Found screen. Use the shift or ctrl keys to select each specimen you want to place in the assay clicking on the specimens in the order you want to add them to the template or use the buttons at the bottom of the Specimens Found screen. After selecting specimens, click the Add to Plate button at the bottom of the screen. LDMS will automatically move to the Plate Preview screen and load the chosen specimens onto the plate in the order determined by the user.
8.2.12 Repeat the process from step six if more than one group needs to be put on the run.
8.2.13 In the Plate Preview screen the user can add and delete plates to run; move, delete add and modify specimens; save the template to run later; click the Run Now button to run the assay.
8.2.14 After saving the assay OD's from the LDMS Remote Reader Software, it can be read into the LDMS database and merged with the template previously set up. Put the disk into the A: drive. Go to the Assay module again.
8.2.15 Click the Viral Load RNA. Highlight the Roche Ultrasensitive (Kit Control) Assay.
8.2.16 Click on the Runs Not Performed button.
8.2.17 Enter the run id of your assay in the Run Id field and click the Search button.
8.2.18 Click on your assay to select it, and then click on the Select button at the bottom right of screen. The Preview tab will activate.
8.2.19 Click on the Preview tab, and then click the Run Now button the bottom of the screen.
8.2.20 The Enter Run Information dialog box will appear. Select the correct kit and the control lot numbers and ranges will be uploaded. Enter tech initials and dates for each step of the assay.
8.2.21 A file dialog box will appear. If using remote read: Open the A:/devdata folder, and then click on your file. Otherwise go the drive on the computer that has the devdata folder.
8.2.22 Click on the Open button on the File dialog box, and LDMS will begin reading the raw data from the disk in your A: drive.

8.2.23 When the LDMS is finished reading the assay data from your remote reader disk of hard drive, the Results screen will appear in Plate Results view displaying the raw data from your remote assay run. Click on the Calculated Results button to view results on a specimen by specimen basis.

8.3 RESULT CRITERIA

8.3.1 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.

8.3.2 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 1:100 dilution with HIV negative human plasma. Calculate the results as above and multiply by100.

8.3.3 If the HIV wells do not follow the pattern of decreasing OD values from well A to well F, and error in dilution may have occurred. Examine the data according to the following criteria to determine if an error occurred. If an error occurred, repeat the entire assay including specimen preparation; otherwise, calculate and report the result as described above:

8.3.3.1 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), expect for well that are saturated and wells with background OD values.

8.3.3.2 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.

8.3.3.3 In reactions containing low HIV-1 RNA copies per ml, wells B through F may contain background OD values. Such tests are valid even thought he HIV wells do not have decreasing OD values from well A through F. Wells with OD values (>2.3) may be saturated and wells with very low OD values (<0.1) are close to background. These wells may not follow a pattern of decreasing OD values from well A to well F.

8.3.4 All well with OD values </=2.3 and >/=0.1 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.

8.3.5 If both QS wells have OD values less than 0.300, either the processed sample was inhibitory to the amplification, or the RNA was no recovered from the sample. The result for that specimen is invalid. Repeat the entire test procedure including specimen processing.

8.3.6 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.

8.3.7 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.
8.3.8 OD dilutions in the 0.2 – 2.0 working range that are less than 3 fold or greater than 7 fold must be redetected.
8.3.9 Undetectable samples greater than the cutoff (standard assay is 400) re-extracting, re-amplifying, and re-detecting.
8.3.10 Any out of sequence errors in the 0.2 – 2.0 range need to be re-detected
8.3.11 Samples with OD ratio failures must be re-detected.
8.3.12 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	0.161	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

8.4 LDMS Specific Criteria

8.4.1 OD ratio errors[16] on a control will trigger an LDMS run censor of D and invalidate the run. If the control is re-detected and found to be valid, the run can be re-marked as valid by using the C user run censor. To apply the C user run censor, click on the Preview Tab and right click on the template. Select Censor Run and highlight the row for the C user run censor. You will be prompted to enter the runid and plate information about the control that was re-detected. Patient reports will not print for specimens on an invalid run until the run is re-marked as valid.
8.4.2 Out-of-sequence errors will trigger an LDMS system censor of L for the control and an LDMS run censor of X3, which will invalidate the run. A control that is out-of-sequence can be re-detected. If the control is re-detected and found to be valid, the run can be re-marked as valid by re-selecting a well through the options button. Patient reports will not print for specimens on an invalid run until the run is re-marked as valid.
8.4.3 False positive results[17], or control out of range will trigger an LDMS run censor of F and will invalidate the run. If the F censor is applied in the absence of OD ratios or out-of-sequence errors, the control cannot be re-detected. No patient reports can be printed and the run must be repeated.
8.4.4 If a control is censored in the LDMS with a DF or X3F, the control can be re-detected. Upon re-detection, if the control is considered valid, the run can be re-marked using the C user run censor (to correct the D censor code) or by re-selecting a well through the options button (to correct the X3 censor code). Patient reports will not print for specimens on an invalid run until the run is re-marked as valid.

9.0 REPORTING RESULTS:

9.1 Interpretation of ACTG Results

Each protocol may have different cutoffs for high and low values decided by various protocol virologists. The LDMS program will print out fax result sheets for either research or clinical purposes.

10.0 PROCEDURE NOTES:

10.1 Heparin should not be used as an anticoagulant as it inhibits PCR.
10.2 Residual ethanol left on the pellet will inhibit the amplification.
10.3 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.
10.4 All reagents should be closely monitored for purity. Discard any reagents that may be suspect.
10.5 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.
10.6 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.
10.7 Gloves must be worn in each area and changed before leaving that area.
10.8 Good laboratory technique is essential to the proper performance of the assay.
10.9 Elevated levels of lipids, bilirubin, total protein, and hemoglobin in specimens do NOT interfere with the quantitation.

11.0 LIMITATIONS OF PROCEDURE:

11.1 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.
11.2 Use of this product should be limited to personnel trained in the techniques of PCR.
11.3 Only the Applied Biosystems Perkin-Elmer GeneAmp PCR System 9600 or GeneAmp 2400 thermal cyclers can be used with this product.
11.4 The AMPLICOR HIV-1 MONITOR test is an in vitro nucleic acid amplification test for the quantification of Human Immunodeficiency Virus Type 1 (HIV-1) RNA in human plasma. The test can quantitate HIV-1 RNA over the range of 50 – 750,000 copies/mL by using a combination of two specimen processing procedures, the Standard and UltraSensitive procedures. With the ultra sensitive procedure, the Test can accurately detect a 0.39 $\log_{10}$(2.5fold) or greater change in HIV-1 RNA if the viral load is 75 – 100,000copies/mL and a 0.68 $\log_{10}$(5-fold) if greater change in HIV-1 RNA if the viral load is approximately 50 copies/mL.
11.5 Reliable results are dependent on adequate specimen collection, transport, storage, and processing procedures.
11.6 This test has been validated for use only with human plasma anticoagulated with EDTA or ACD. Heparin inhibits PCR.
11.7 As with any diagnostic test, results from the AMPLICOR HIV-1 MONITOR test should be interpreted with consideration of all clinical and laboratory findings.

11.8 When compared with the version 1.0 assay, the version 1.5 demonstrated improved detection efficiency for HIV-1 group M, sub-types A-H. However the linearity, reproducibility, and limit of detection have not been evaluated for non B subtypes.

11.9 This assay is not intended to be used as a screening test for HIV or as a diagnostic test to confirm the presence of HIV infection.

11.10 The AMPLICOR HIV-1 MONITOR test version 1.5 appears to have a higher variation compared to version 1.0

11.11 It is recommended the user re-establish the baseline viral load level for non B subtypes. Studies have demonstrated that version 1.5 produces results that are comparable or enhanced from results generated from the previous version.

12.0 PERFORMANCE CHARACTERISTICS:

12.1 Limit of Detection, Linear Range and Precision

The analytic performance of the AMPLICOR HIV-1 MONITOR Test, version 1.5 using the Standard and UltraSensitive specimen preparation procedure was determined by testing serial dilutions of a well characterized stock of cultured HIV-1 virus into HIV-negative human plasma. The virus stock, a HIV-1 group M subtype B isolate, was provided by the Virology Quality Assurance (VQA) Laboratory of the AIDS Clinical Trials Group' (ACTG), Division of AIDS, National Institutes of Health, Bethesda, MD, USA. The concentration of viral RNA in the virus stock was estimated by electron microscopy, p24 antigen concentration, the AMPLICOR HIV-1 MONITOR Test, and branched chain DNA Analysis.

These panels were tested in multi-sites with multi-operator (2 within sites) and multi-reagent lots (3). The study was carried out for 8 days per site, per lot, assaying 3 aliquots of each panel member each day.

12.2 Limit of Detection

Limit of detection is defined as the viral concentration at which the sample can be detected as reactive at a rate of 95% or better. The data from this study are shown in Table 2 for the Standard specimen preparation procedure and Table 3 for the UltraSensitive specimen preparation procedure.

Table 2

Limit of Detection, Standard Specimen Preparation

HIV-1 RNA (copies/mL)	Positive Results	Negative Results	Sensitivity	(95% CI)
175	98	26	0.79	0.71, 0.86
250	101	20	0.83	0.76, 0.90
400	115	7	0.94	0.89, 0.98
500	119	2	0.98	0.94, 1.00
600	121	4	0.97	0.92, 0.99
2000	113	0	1.00	0.97, 1.00
7000	125	0	1.00	0.97, 1.00
35000	123	0	1.00	0.97, 1.00
100000	124	0	1.00	0.97, 1.00
350000	121	0	1.00	0.97, 1.00
500000	126	0	1.00	0.97, 1.00
650000	119	0	1.00	0.97, 1.00

750000	126	0	1.00	0.97, 1.00
850000	122	0	1.00	0.97, 1.00

Table 3

Limit of Detection, UltraSensitive Specimen Preparation

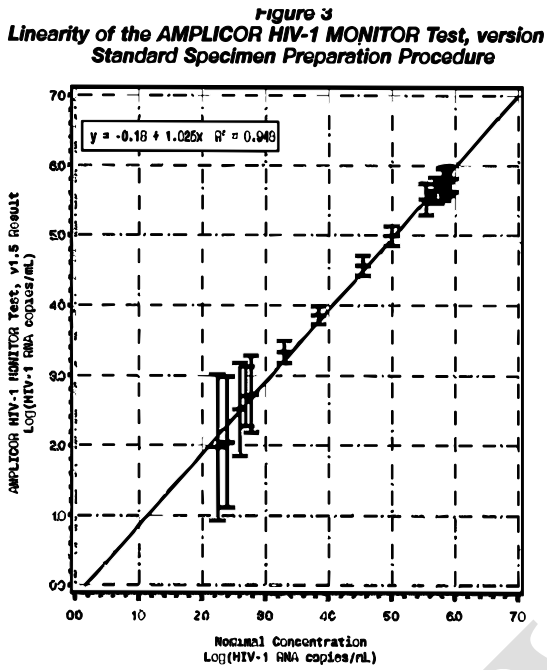
HIV-1 RNA (copies/mL)	Positive Results	Negative Results	Sensitivity	(95% CI)
25	86	17	0.83	0.75, 0.090
40	102	3	0.97	0.92, 0.99
50	101	2	0.98	0.93, 1.00
60	106	0	1.00	0.97, 1.00
80	104	1	0.99	0.95, 1.00
100	103	0	1.00	0.96, 1.00
400	108	0	1.00	0.97, 1.00
1000	112	0	1.00	0.97, 1.00
5000	114	0	1.00	0.97, 1.00
25000	110	0	1.00	0.97, 1.00
35000	112	0	1.00	0.97, 1.00
50000	110	0	1.00	0.97, 1.00
75000	112	0	1.00	0.97, 1.00
100000	112	0	1.00	0.97, 1.00

These results show that the limit of detection of the version 1.5 assay for the Standard specimen preparation procedure is 400 HIV-1 RNA copies/mL. For the UltraSensitive specimen preparation procedure, the estimated limit of detection is 50 HIV-1 RNA copies/mL.

12.3 Linear Range

The linear range was determined by regression analysis using expected HIV-1 ANA concentrations and the corresponding test results.

Figure 3
Linearity of the AMPUCOR HIV-1 MONITOR Test, version 1.5
Standard Specimen Preparation Procedure

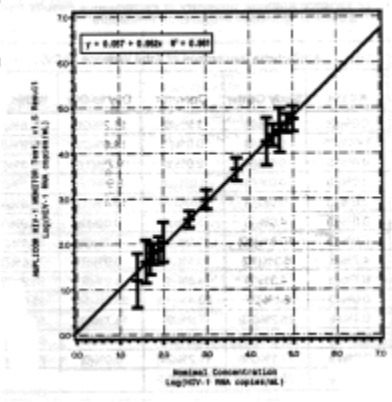


As shown in Figure 3, the AMPLICOR HIV-1 MONITOR Test, version 1.5 with Standard specimen processing was found to give a linear response from 400 (log10 = 2.60) to at least 750,000 (log10 = 5.85) HIV-1 RNA copies/mL and with UltraSensitive specimen preparation was found to give a linear response from 50 (log10 = 1.70) to at least 100,000 (log10 = 5.00) HIV-1 RNA copies/mL (see Figure 4). Patients with viral load results less than 100,000 HIV-1 RNA copies/mL should be monitored using the UltraSensitive specimen preparation procedure.

Figure 4

Linearity of the AMPL/COR HIV-1 MONITOR Test, version 1.5

UltraSensitive Specimen Preparation Procedure



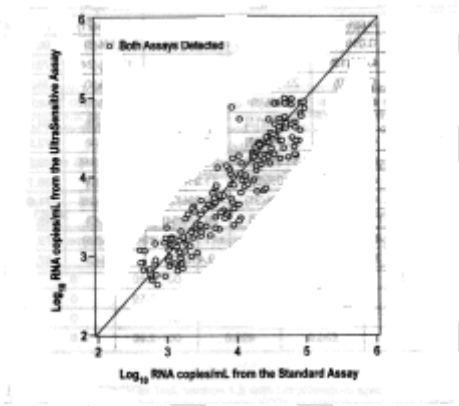
12.4 CORRELATION OF RESULTS FOR STANDARD AND ULTRASENSITIVE SPECIMEN PREPARATION PROCEDURES

In studies conducted at three separate laboratories, 181 patient specimens were tested by the AMPLICOR HIV-1 MONITOR Test, version 1.5 using both the Standard and UltraSensitive specimen preparation procedures. The overall correlation for results combined across laboratories was 0.932 with a bias towards lower values (0.0029 Log10 lower by the UltraSensitive procedure).

Figure 5

AMPL/COR HIV-1 MONITOR Test, version 1.5 Correlation of Standard and

UitraSensitive Specimen Preparation Procedures



12.5 Precision

For the Standard specimen preparation procedure of the AMPLICOR HIV-1 MONITOR Test, version 1.5, Coefficients of Variation (CV's) in the linear range were between 30% and 94%, while the prior version of the assay had CV's of between 32.3% and 45.3%. For the UltraSensitive specimen preparation procedure of the version 1.5 assay, the CV's over the linear assay range of the assay were 32% to 102%, compared to CV's between 30.2% and 41.9% for the prior version.

Table 4

Precision of the AMPL/COR HIV-1 MONITOR Test, version 1.5, Components of Variance Analysis; Summary of Performance Results from S1andard Specimen Preparation Procedure

Input HIV-1 RNA (copies/mL)	Total Standard Deviation (copies/mL)	Attribution Percentage of Total Variance (%CV)					
		Kit lot	Study Center	Operator	Day to Day	Within Run	Total CV
175	236	0.0% (0)	0.0% (0)	1.2% (8)	11.2% (25)	87.6% (69)	74%
250	163	1.64% (7)	0.0% (0)	0.% (0)	14.6% (22)	83.9% (52)	56%
400	277	0.0% (0)	0.0% (0)	2.0% (8)	9.7% (17)	88.3% (50)	54%
500	389	0.0% (0)	0.0% (0)	6.9% (16)	6.9% (16)	86.3%(57)	61%
600	380	0.0% (0)	0.0% (0)	3.8% (10)	10.4% (17)	85.8% (48)	52%
2000	826	0.0% (0)	4.0% (7)	5.1% (8)	0.0% (0)	90.9% (34)	36%
7000	2294	0.0% (0)	10.8% (10)	0.0% (0)	9.2% (9)	80.1% (27)	30%
35000	14841	4.7% (8)	6.3% (10)	10.5% (12)	5.9% (9)	72.6% (33)	38%
100000	40791	0.7% (8)	4.3% (7)	4.6% (8)	4.9% (9)	86.2% (36)	39%
350000	372502	0.0% (0)	8.1% (27)	23.4% (46)	33.5% (55)	35.0% (56)	94%

500000	328155	0.4% (4)	25.2% (33)	1.9% (9)	15.7% (26)	56.8% (50)	66%
650000	497804	0.0% (0)	21.5% (37)	9.3% (24)	6.7% (20)	62.5% (62)	79%
750000	481108	0.0% (0)	17.1% (29)	11.2% (24)	0.0% (0)	71.7% (60)	71%
850000	356316	0.0% (0)	26.5% (25)	21.6% (23)	0.0% (0)	51.9% (35)	49%

Table 5

Precision of the AMPL/COR HIV-1 MONITOR Test, *version* 1.5, *Components of Variance Analysis; Summary of Performance Results from UltraSensitive Specimen Preparation Procedure*

Input HIV-1 RNA (copies/mL)	Total Standard Deviation (copies/mL)	Attribution Percentage of Total Variance (%CV)					
		Kit lot	Study Center	Operator	Day to Day	Within Run	Total CV
25	28	0.0% (0)	0.0% (0)	0.1% (3)	7.1% (24)	92.8% (86)	90%
40	20	0.0% (0)	0.0% (0)	0.0% (0)	20.0% (21)	80.0% (42)	47%
50	31	0.0% (0)	0.0% (0)	7.3% (15)	2.1% (8)	90.6% (52)	54%
60	44	4.4% (13)	1.3% (7)	0.0% (0)	15.3% (24)	79.0% (55)	62%
80	42	0.0% (0)	23.9% (24)	0.0% (0)	0.8% (4)	75.2% (42)	49%
100	48	0.0% (0)	1.6% (6)	1.0% (5)	0.6% (3)	96.8% (45)	46%
400	122	7.0% (8)	22.7% (15)	0.0% (0)	6.1% (8)	64.3% (26)	32%
1000	360	0.0% (0)	25.7% (18)	0.0% (0)	1.4% (4)	72.9% (31)	36%
5000	5694	0.0% (0)	33.9% (60)	0.0% (0)	22.8% (49)	43.3% (67)	102%
25000	22389	0.0% (0)	0.0% (0)	11.9% (31)	0.0% (0)	88.1% (83)	89%
35000	22848	0.0% (0)	16.7% (31)	0.0% (0)	11.7% (26)	71.6% (64)	75%
50000	39302	11.9% (31)	4.9% (20)	36.6% (54)	0.0% (0)	46.6% (61)	90%
75000	27265	0.0% (0)	10.2% (17)	1.7% (7)	8.2% (15)	79.9% (47)	53%
100000	49993	3.0% (12)	0.0% (0)	9.4% (22)	0.0% (0)	87.6% (67)	72%

12.6 Analytical Specificity

The analytical specificity of the AMPLICOR HIV-1 MONITOR Test, version 1.5 was evaluated by adding cultured cells, cultured virus or purified nucleic acid from the following organisms and viruses into HIV negative human plasma (after Step 81.6 of the *"Instructions for* Use"), then analyzing these samples. None of the non-HIV organisms, viruses or purified nucleic acids tested showed reactivity in the AMPLICOR HIV-1 MONITOR Test, version 1.5 when performing the Standard specimen preparation procedure. Two of the four HIV-2 isolates tested (7824A and 60415K) yielded positive results; however, no specific claims can be made for the ability of this test to amplify HIV-2 isolates (Table 6).

Table 6

List of Organisms, Cultured Virus, or Purified Nucleic Acid Tested with the AMPLICOR HIV-1 MONITOR Test, version 1.5

Adenovirus type 2	Hepatitis C Virus Genotype 4c	Human Herpes simplex type I, strain Macintyre
Adenovirus type 3	HIV-2, subtype A/B* (Isolate 7312A)	Human papilloma virus 6b
Adenovirus type 7	HIV-2, subtype A* (Isolate 7824A)	Human papilloma virus 11
Candida albicans	HIV-2, BEN*	Human papilloma virus 16

Cytomegalovirus AD-169	HIV-2, subtype A* (ISOlate 6O415K)	Human papilloma virus 18
Cytomegalovirus Davis	HTLV-1, C5/MJ cells	*Mycobacterium avium*
Cytomegalovirus Towne	hTLV-1, 0S-P2 cells	*Pneumocystis carinii*
Epstein-Barr virus (RAJI- Burkitt's Lymphoma cells)	HTLV-I, MT-2 cells	*Propionibacterium acnes*
Epstein-Ban virus HR1 (Burkitt's Lymphoma cells)	HTLV-I, MJ cells	*Staphylococcus aureus*
Epstein-Ban virus P-3 (Burkitt's Lymphoma cells)	HTLV-II, MoT cells	*Staphylococcus epidermidis*
Hepatitis B Virus *(full length clone)*	HTLV-II (full length clone)	*Varicella-Zoster Ellen*
Hepatitis B Virus (patient sera)	Human Herpes Virus 6	*Varicella Oka*
Hepatitis C Virus Genotype 1 a	Human Herpes Virus 7	
Hepatitis C Virus Genotype 1b	Herpes simplex type I, strain F	
Hepatitis C Virus Genotype 2a/2c	Human Herpes simplex type I, strain HF	
Hepatitis C Virus Genotype 2b	Human Herpes simplex type II, Strain MS	
Hepatitis C Virus Genotype 3a	Human Herpes simplex type II, strain G	

*2 of 4 HIV-2 isolates yielded positive results.

12.7 CLINICAL SPECIFICITY

Standard Specimen Preparation

The clinical specificity of the AMPLICOR HIV-1 MONITOR Test, version 1.5 with Standard specimen preparation was determined by analysis of 459 specimens from anti-HIV-1 negative blood donors. Specimens containing either EDTA or ACD as anticoagulant were tested with three lots of the AMPLICOR HIV-1 MONITOR Test, version 1.5. None of these specimens were reactive with the AMPLICOR HIV-1 MONITOR Test, version 1.5 (Table 7). Assuming a zero prevalence of HIV-1 infection in the seronegative blood donors, the specificity of the test was 100% [95% CI = (99.2-100%)].

Table 7
Anti-HIV-1 Negative Blood Donors Specificity Analysis of *Serology Negative Donor specimens, Standard Specimen Preparation Procedure*

HIV-1 RNA				Distribution of Abosrbency (A_{450}) Values				
Kit Lot #	Not Detected	Detected	95% CI	Minimum	Average	Maximum	SD	%CV
1	84	0	95.7-100	0.048	0.062	0.093	0.010	17
2	146	0	97.5-100	0.047	0.067	0.132	0.017	26
3	229	0	98.4-100	0.029	0.059	0.160	0.017	28

Overall	459	0	99.2-100	0.029	0.062	0.160	0.016	26

UltraSensitive Specimen Preparation

The clinical specificity of the AMPLICOR HIV-1 MONITOR Test, version 1.5 with UltraSensitive specimen preparation was determined by analysis of 457 specimens from anti-HIV-1 negative blood donors. Specimens containing EDTA as anticoagulant were tested with three kit lots of the AMPLICOR HIV-1 MONITOR Test, version 1.5. None of the specimens were reactive with the AMPLICOR HIV-1 MONITOR Test, version 1.5 (Table 8). Assuming a zero prevalence of HIV-1 infection in the seronegative blood donors, the specificity of the test was 100% [95% CI = (99.2-100)].

Table 8
Anti-HIV-1 Negative Blood Donors Specificity Analysis of *Serology Negative Donor* specimens, *UltraSensitive Specimen Preparation Procedure*

HIV-1 RNA				Distribution of Abosrbency (A_{450}) Values				
Kit Lot #	**Not Detected**	**Detected**	**95% CI**	**Minimum**	**Average**	**Maximum**	**SD**	**%CV**
1	8123	0	97.1-100	0.047	0.063	0.112	0.013	21
2	125	0	97.1-100	0.047	0.071	0.130	0.015	22
3	209	0	98.3-100	0.031	0.061	0.121	0.017	27
Overall	457	0	99.2-100	0.031	0.064	0.130	0.016	25

12.8 INCLUSIVITY: PERFORMANCE OF HIV-1 GROUP M Non-B Subtypes

The primary purpose for developing the AMPLICOR HIV-1 MONITOR Test, version 1.5 was to improve the detection efficiency of Group M non-B subtypes as compared to the version 1.0 assay. Three studies were conducted to evaluate the performance of the version 1.5 on HIV-1 group M subtypes A to H.

12.8.1 Study 1

Study material for 14 of 16 isolates used in this study came from 30 HIV-1 isolates of known HIV-1 Group M subtypes that were cultured and characterized by electron microscopic particle counting, infectious titer, p24 Ag concentration, and reverse transcriptase activities. These virus stocks were prepared in collaboration with Walter Reed Army Institute of Research. The remaining two isolates are part of a panel of 44 HIV-1 isolates of known subtype that were cultured and characterized by electron microscopic particle counting as part of an effort by AIDS Clinical Trial Group (ACTG) Virology Quality Assurance Program to produce a set of international HIV-1 standards. This panel includes all 30 isolates in the Roche-Walter Reed panel.

Sixteen panels were prepared from different HIV-1 isolates of subtypes A, B, C, D, E, F, G and H. Each panel consisted of 7 concentrations. The study was carried out at three sites. Each site received the panels with blinded numbers and each panel (n=7 Standard and 7 UltraSensitive Specimen Preparation panel members) was tested using 3 manufacturing lots of reagents.

The mean results for all concentration levels of all panel members at each dilution were used to compare the performance of version 1.5 to that of version 1.0 for both sample preparation methods. Figures 6 and 7 present the mean test results for the AMPLICOR HIV-1 MONITOR Test, versions 1.5 and 1.0 when using the UltraSensitive specimen preparation procedure. Figures 8 and 9 present the mean test results for the AMPLICOR HIV-1 MONITOR Test, version 1.5 and 1.0, respectively, when the Standard specimen preparation procedure was used. Examination of these results indicate that, for both

specimen preparation procedures, with all Group M, non-B isolates, the AMPLICOR HIV-1 MONITOR Test, version 1.5 demonstrates improved performance compared to version 1.0 of the assay.

These results show that the AMPLICOR HIV-1 MONITOR Test, version 1.5 provides comparable or enhanced performance in amplification of specimens containing HIV-1 Group M, subtype A-H when compared with version 1.0 of the same assay.

Figure 6 *Performance of AMPLICOR HIV-1 MONITOR* Test, *version* 1.5 *on HIV-1 Subtypes* A *through H, when using* the *U/fraSensitive Specimen Preparation Procedure*

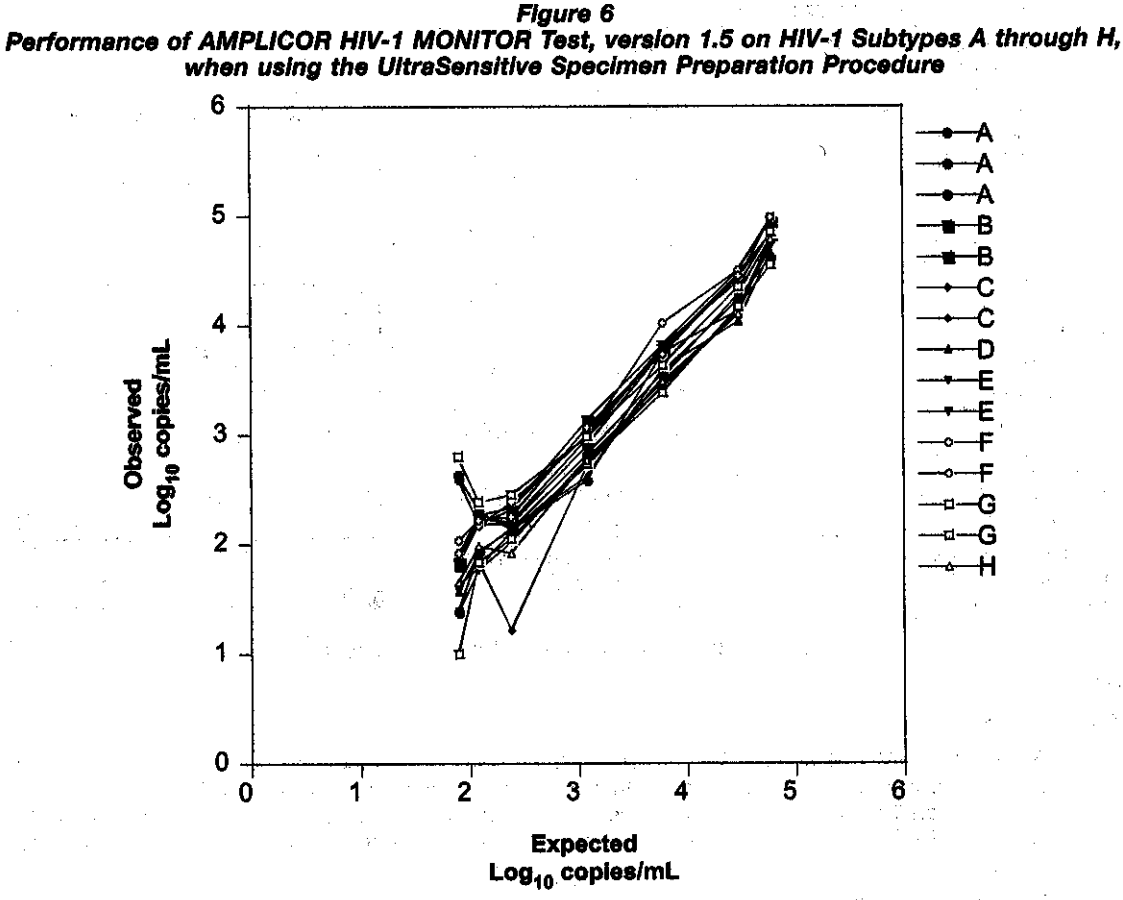


Figure 7 *Performance of AMPLICOR HIV-1 MONITOR* Test, *version 1.0* on *HIV-1 Subtypes* A *through H, when using* the *UltraSensitive Specimen Preparation Procedure*

Figure 7
Performance of AMPLICOR HIV-1 MONITOR Test, version 1.0 on HIV-1 Subtypes A through H, when using the UltraSensitive Specimen Preparation Procedure

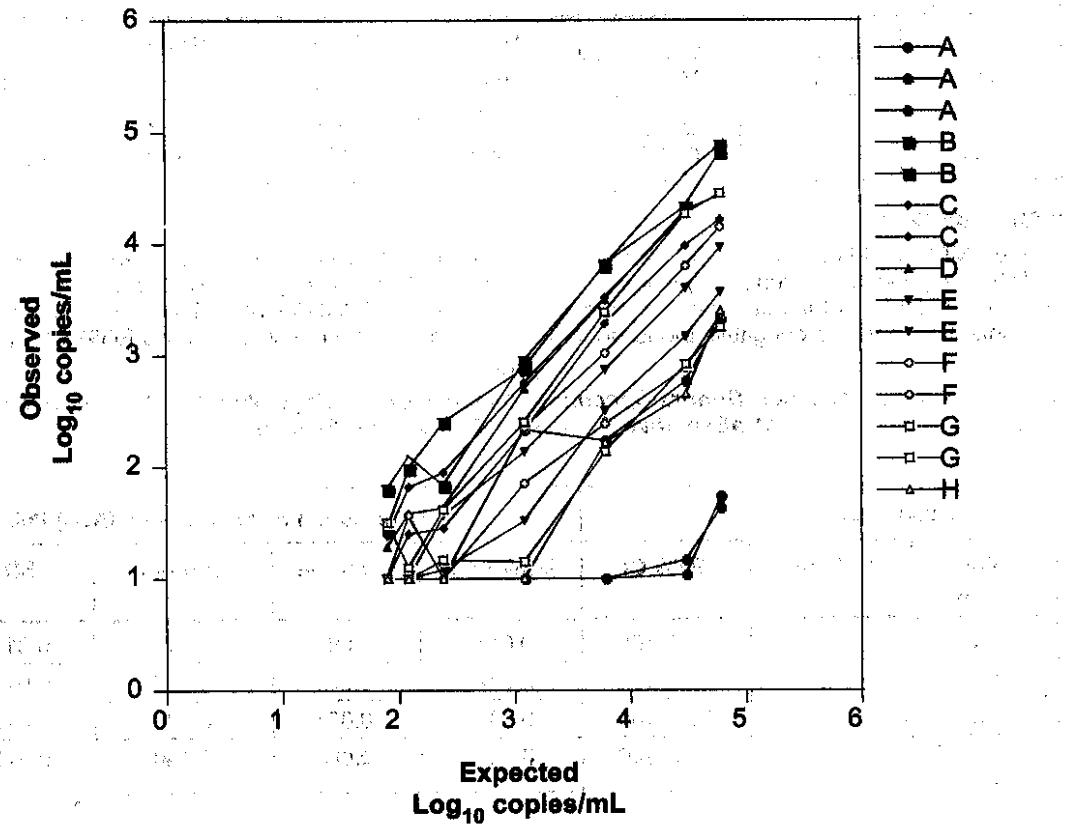


Figure 8
Performance of AMPLiCOR HIV-1 MONITOR Test, *version* 1.5 *on HIV-1 Subtypes* A *through H, when using the Standard Specimen Preparation Procedure*

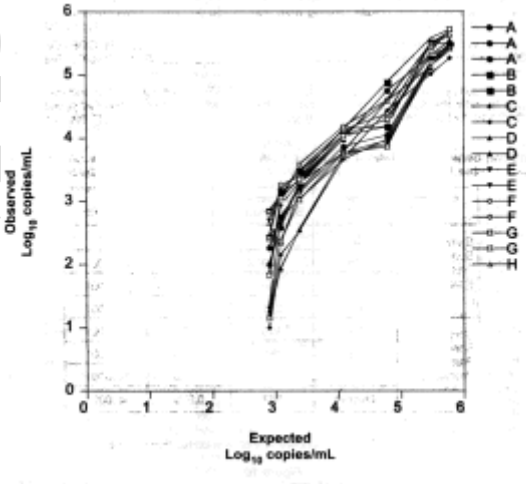
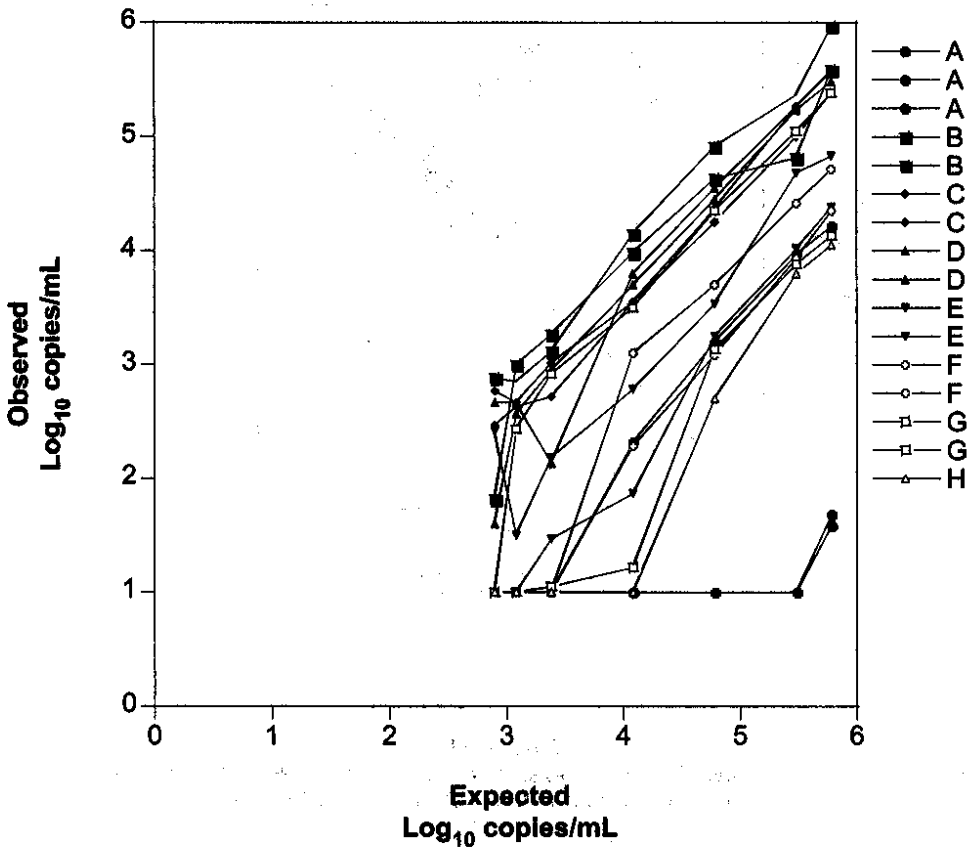


Figure 9 *Performance of AMPLiCOR HIV-1 MONITOR Test, version 1.0 on HIV-1 Subtypes* A *through H, when using the Standard Specimen Preparation Procedure*

Figure 9
Performance of AMPLICOR HIV-1 MONITOR Test, version 1.0 on HIV-1 Subtypes A through H, when using the Standard Specimen Preparation Procedure



12.8.2 Study 2

The performance of the AMPLICOR HIV-1 MONITOR Test, version 1.5 with both the Standard and UltraSensitive specimen processing procedure was further evaluated by assaying the World Wide HIV-1 subtype performance panel (WWRB301) from Boston Biomedica, Inc. The panel consists of fifty, plasma specimens from HIV-1 infected patients from Africa, Asia, South America and North America. This well characterized panel contained data on the subtype of each HIV-1 infection, viral load results (Chiron Quantiplex, Organon Teknika NASBA, AMPLICOR HIV-1 MONITOR Test, version *1.0),* and serology test results.

In this study three trial sites participated, analyzing all 50 members of the panel using both sample preparation methods.

Examination of these data suggests that the AMPLICOR HIV-1 MONITOR, version 1.5 assay, with both the Standard and UltraSensitive specimen preparation procedures provide consistently better results compared to the AMPLICOR HIV-1 MONITOR, version *1.0,* assay,

12.8.3 Study 3

This study was conducted in a clinical setting in the United Kingdom, where patients with HIV-1 Group M, non-B subtype infections were monitored using the AMPLICOR HIV-1 MONITOR Test, version 1.5, The data was collected as part of a clinical audit. Patient inclusion criteria included: 1) having infection with HIV-1 group M non-B subtype, as defined by serologic testing and partial sequences of the HIV-1 genome, and 2) having been tested at 5 or more time points using the AMPLICOR HIV-1 MONITOR Test, version 1.5 during a period when antiretroviral drug therapy was initiated or switched. Twenty eight (28) of 152 patients met the criteria for inclusion. This subpopulation was composed of 16 males and 12 females. Twenty-six of the patients received a 3 drug (or more) therapy. Upon initiation or switching of drug therapy, 16 of these patients had at least one viral concentration below the limit of detection while the remaining 12 patient's viral load dropped to an average of 19,800 copies/mL during the study. All those patients who did not achieve undetectable viral loads in response to therapy were noted to have adherence problems or were on suboptimal therapy. These results indicate the AMPLICOR HIV-1 MONITOR Test, version 1.5 was useful in monitoring patients infected with HIV-1 Group M non-B subtypes for antiretroviral drug therapy.

12.9 Performance of the AMPLICOR HIV-1 MONITOR Test, version 1.5, Compared to the AMPLICOR HIV-1 MONITOR Test, version 1.0

The performance of the AMPLICOR HIV-1 MONITOR Test, version 1.5 was compared to that of the AMPLICOR HIV-1 MONITOR Test, version 1.0 by testing archived HIV-1 reactive plasma samples with prior AMPLICOR HIV-1 MONITOR, version 1.0 test results. All samples were collected in the United States and were presumed to be subtype B. Three hundred seventy one (371) such samples with AMPLICOR HIV-1 MONITOR Test, version 1.0 Standard specimen preparation procedure test results ranging from 400 to 750,000 HIV-1 RNA copies/mL were tested using the AMPLICOR HIV-1 MONITOR Test, version 1.5 Standard specimen preparation procedure. The data are presented in Figure 10.

Similarly, three hundred forty seven (347) archived HIV-1 reactive samples with prior UltraSensitive AMPLICOR HIV-1 MONITOR Test. Version 1.0 test results ranging from 0 to 75,000 copies/mL were analyzed at three sites with the AMPLICOR HIV-1 MONITOR Test, version 1.5 UltraSensitive specimen preparation procedure. The data are presented in Figure 11.

As summarized in Table 9, the test results obtained with the AMPLICOR HIV-1 MONITOR Test, version 1.5 and the AMPLICOR HIV-1 MONITOR Test, version 1.0 are highly correlated for both the Standard specimen preparation procedure and UltraSensitive specimen preparation procedure. Thus, the test results of the AMPLICOR HIV-1 MONITOR Test, version 1.0 and the AMPLICOR HIV-1 MONITOR Test, version 1.5 were comparable.

Table 9

Correlation between the AMPLICOR HIV-1 MONITOR Tes1 Assays, versions 1.0 and 1.5 with the Standard and UltraSensitive Specimen Preparation Procedures

Method	Site	# of specimens	Correlation	P Value

Standard Specimen Preparation Procedure	04	113	0.942	<.0001
	05	113	0.972	<.0001
	06	145	0.965	<.0001
	Overall	371	0.961	<.0001
UltraSensitive Specimen Preparation Procedure	04	116	0.980	<.0001
	05	98	0.976	<.0001
	06	133	0.976	<.0001
	Overall	347	0.976	<.0001

1 *Correlation by Spearman's coefficient of rank correlation method.*

Figure 10

Correlation of the AMPLICOR HIV-1 MONITOR Test, version 1.5 and the AMPLICOR HIV-1 MONITOR Test, version 1.0 on Plasma Specimens from HIV-1 Infected Patients

Standard Specimen Preparation Procedure

Figure 10
Correlation of the AMPLICOR HIV-1 MONITOR Test, version 1.5 and the AMPLICOR HIV-1 MONITOR Test, version 1.0 on Plasma Specimens from HIV-1 Infected Patients

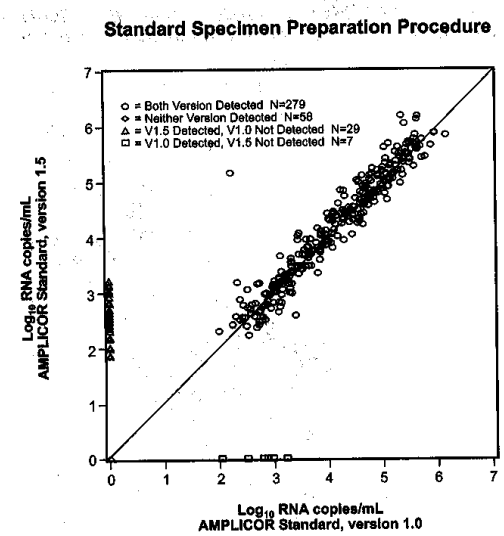
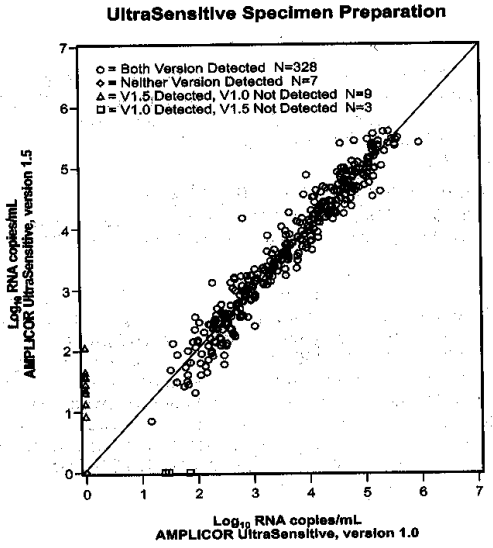


Figure 11

Correlation of the AMPLICOR HIV-1 MONITOR Test, version 1.5 *and the AMPLICOR HIV-1 MONITOR Test, version 1.0 on Clinical Specimens from HIV-1Infected Subjects*

UltraSensitive Specimen Preparation

Figure 11
Correlation of the AMPLICOR HIV-1 MONITOR Test, version 1.5 and the AMPLICOR HIV-1 MONITOR Test, version 1.0 on Clinical Specimens from HIV-1 Infected Subjects



12.10 CLINICAL PERFORMANCE

The clinical utility of the AMPLICOR HIV-1 MONITOR Test was demonstrated using the version 1.0 test only. Since the analytical performance for the AMPLICOR HIV-1 MONITOR Test, version 1.5, is similar to or better than that of version 1.0, the data presented here for version 1.0 are expected to be applicable to version 1.5.

12.10.1 Patient Prognosis

The 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. ACTG Study 116A was a double-blinded study that compared the clinical efficacy of zidovudine (ZDV) in combination with two doses of 2',3'dideoxyinosine (ddI) 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 2',3'dideoxyinosine (ddI) and zidovudine therapy of patients with HIV infection who had been on zidovudine 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, a new AIDS defining event, or death.

The ACTG 116A HIV-1 RNA sub-study included 186 patients from the clinical study who had had plasma and PBMC specimens collected upon entrance into the study and whose specimens had been stored appropriately and were available for testing. The ACTG 116B/117 HIV-1 RNA sub-study included 99 randomly selected patients from the drug study who had plasma specimens collected on entrance into the study and whose specimens had been stored appropriately and were available for testing.

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 Cox Proportional Hazards Models. The unadjusted Relative Hazard represents the risk conferred by the variable alone, whereas the Adjusted Relative Hazard represents the risk conferred by that variable after controlling for the other variables in the model. These models give the increased risk (if any) of disease progression associated with the variables entered into the model. These analyses were performed by assessing the relative hazards of a 5-fold difference in the study variable. The results of these analyses are presented in Tables 10 through 14. These data show that in a 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 of prior ZDV therapy (patients in ACTG Study 116B/117), 5-fold higher baseline HIV-1 RNA levels were not of statistically significant prognostic value. For patients who have had either no prior ZDV therapy or 16 weeks or less of ZDV therapy, 5-fold changes between baseline and Week 8 RNA levels have statistically 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. The frequency of disease progression was also analyzed for each study by dividing each study population into deciles by rank order of base- line HIV-1 RNA. The deciles were evaluated for the frequency of disease progression. For each study, a frequency of disease progression of 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, < 34,661, < 72,438 and < 1 03,806 HIV-1 RNA copies/mL). The frequency of disease progression was between 40% and 50% in the nex1 three deciles « 150695, < 194,312 and < 247,229). 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 (<11,571, < 31,292, < 49,743, < 62132, < 97,781 and < 150,866). The rate of disease progression increased to 50% and 60% for the next two deciles

(<251,627 and < 403,146, respectively). The disease progression rates were greater than 60% for the last two deciles (< 794,027 and < 1,456,302) where the HIV-1 RNA levels were greater than 4O3,000. Summary tables and bar charts of these analyses are presented in Figures 12 and 13.

Table 10

Association of Study Variables at Baseline with Disease Progression ACTG Study 116A (N = 153 *Patients,* 73 *Progression Events)*

Variable	Unadjusted Relative Hazard (95% CI)	Adjusted Relative Hazard (95% CI)	p value2
Log HIV-1 RNA copy number	1.58 (1.20 -2.09)	1.44 (1.07 -1.93)	0.02
Log CD4+ cell count	0.39 (0.28 -0.54)	0.45 (0.31 -0.64)	0.0001
Dx of AIDS at Baseline	2.00 (1.22 -3.27)	1.39 (0.82 -2.37)	0.22
ddl Treatment	0.95 (0.59 -1.53)	1.12 (0.68 -1.64)	0.66

1 -The Relative Hazard is the hazard ratio resulting *from* a 5-fold increase in HIV-1-RNA or decrease in CD4+ cell count

2 -p values are for Adjusted Relative Hazards

Table 11

Association of Change in HIM RNA from Baseline to Week 8 with Disease Progression ACTG Study 116A (N = 114 Patients, 62 Progression Events)

Variable	Unadjusted Relative Hazard (95% CI)	Adjusted Relative Hazard (95% CI)	p value2
LogHIV-1RNAcopy number1	1.46(1.11-1.93)	1.63(1.16-2.28)	0.0005
LogChangeinHIV-1RNA *from* Baseline to Week 81	1.18(0.93-1.48)	1.54(1.09-2.16)	0.013
Log CD4+ cell count1	0.43 (0.30 -0.62)	0.50 (0.34 -0.73)	0.0004
Dx of AIDS at Baseline	1.83 (1.09 -3.09)	1.28 (0.74 -2.21)	0.38
ddl Treatment	0.76 (0.45 -1.27)	0.87 (0.51 -1.49)	0.61

1 -The Relative Hazard is the hazard ratio resulting from a 5-fold increase in HIV-1-RNA or decrease in CD4+ cell count

2 -p values are for Adjusted Relative Hazards ~

Table 12

I*Association of Study Variables at Baseline with Disease Progression ACTG Study 116B/117 (N* = 86 *Patients,* 39 *Progression Events)*

Variable	Unadjusted Relative Hazard (95%CI)	Adjusted Relative Hazard (95% CI)	p value[2]
Log HIV-1 RNA copy number1	1.90 (1.28 -2.82)	1.25 (0.81 -1.94)	0.32
Log CD4+ cell count1	0.28 (0.16 -0.48)	0.33 (0.18 -0.62)	0.0006

Dx of AIDS at Baseline	3.13(1.66-5.92)	2.38(1.24-4.58)	0.01
ddl Treatment	0.89 (0.47 -1.69)	0.88 (0.46 -1.71)	0.71

1 -The Relative Hazard is the hazard ratio resulting from a 5-fold increase in HIV-1-RNA or decrease in CD4+ cell count

2'- P values are for Adjusted Relative Hazards

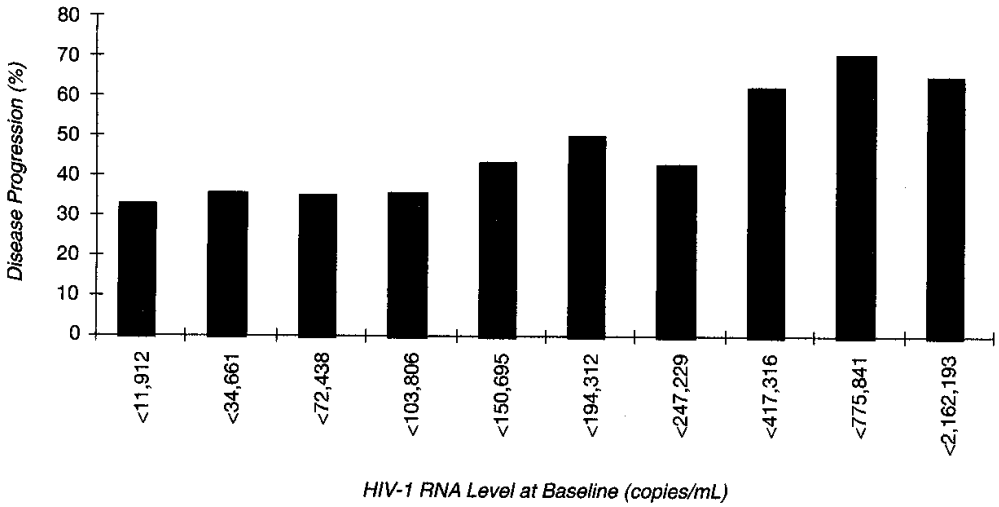
Table 13
Association of Change in HIM RNA from Baseline to Week 8 *with Disease Progression ACTG Study 116B/117 (N =* 65 *Patients,* 29 *Progression Events)*

Variable	Unadjusted Relative Hazard (95%CI)	Adjusted Relative Hazard (95% CI)	p value[2]
LogHIV-1RNAcopy number1	2.10(1.32-3.34)	1.58(0.93-2.69)	0.09
Log Change in HIV-1 RNA from Baseline to Week 81	1.41 (0.71 -2.79)	1.58 (0.68 -3.68)	0.29
Log CD4+ cell count1	0.25 (0.13 -0.46)	0.29 (0.14 -0.60)	0.001
Dx of AIDS at Baseline	2.43(1.17-5.05)	1.87(0.88-3.97)	0.10
ddl Treatment	1.07 (0.51 -2.27)	0.98 (0.39 -2.48)	0.96

1 -The Relative Hazard is the hazard ratio resulting from a 5-fold increase in HIV-1-RNA or decrease in CD4+ cell count

2 -P values are for Adjusted Relative Hazards

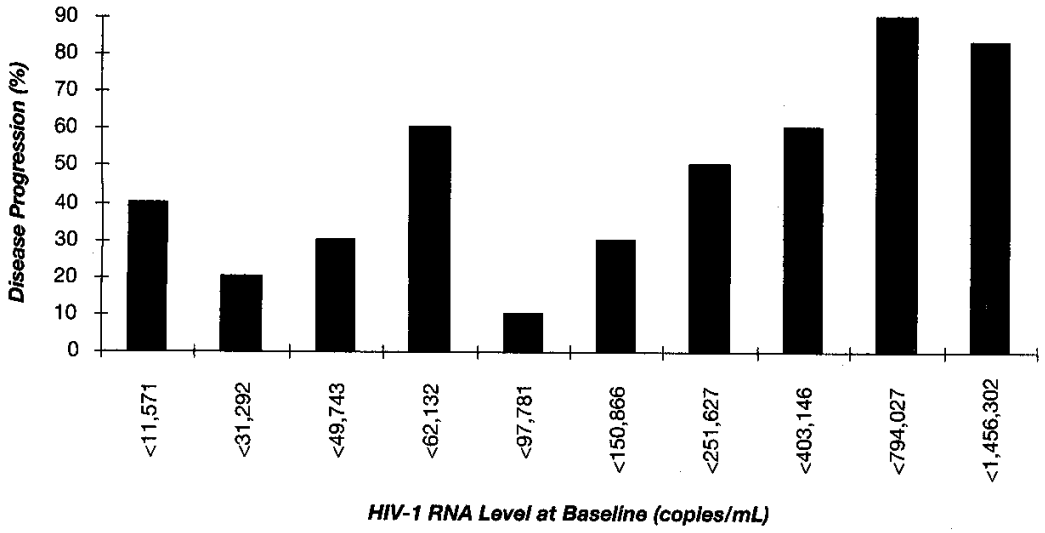
Figure 12
Frequency of Disease Progression by Baseline HIV-1 RNA Level
Study 116A



Record #	Decile	n=	# Progressions	# Progressions to AIDS/Death	%
18	<11912	18	15	5	33.33
36	<34661	18	14	5	35.71
54	<72438	18	17	6	35.29
72	<103806	18	14	5	35.71
90	<150695	18	16	7	43.75

108	<194312	18	16	8	50.00
126	<247229	18	14	6	42.86
144	<417316	18	16	10	62.50
162	<775841	18	17	12	70.59
179	<2162193	17	14	9	64.29

Figure 13
Frequency of Disease Progression by Baseline HIV-1 RNA Level
Study 116B/117



Record #	Decile	n=	# Progressions	# Progressions to AIDS/Death	%
10	<11571	10	10	4	40.00
20	<31292	10	10	2	20.00
30	<49743	10	10	3	30.00
40	<62132	10	10	6	60.00
50	<97781	10	10	1	10.00
60	<150866	10	10	3	30.00
70	<251627	10	10	5	50.00
80	<4031	46	10	10 6	60.00
90	<794027	10	10	9	90.00
96	<456302	6	6	5	83.33

12.10.2 Measuring Response to Antiretroviral Therapv

The use of the AMPLICOR HIV-1 MONITOR Test to measure the effects of antiretroviral therapy was evaluated in a clinical study of antiretroviral compounds including the reverse transcriptase inhibitor zalcitabine (ddC, tradename HIVID), the protease inhibitor drug Saquinavir (SAO, tradename INVIRASE, and combinations of these two drugs. The study (Study NV14256) was a double-blind, phase 111 randomized study whose primary objective was to evaluate the safety, tolerability and efficacy of three treatments (ddC, SAO, and SAO in combination with ddC) based on clinical endpoints in patients discontinuing (or unable to take) zidovudine (ZDV) therapy and to compare survival among the three treatment groups [including death after an AIDS Defining Event (ADE), or dose limiting toxicity].

For Study NV14256, prior ZDV treatment was an inclusion criterion with the majority of patients having over one year of prior ZDV treatment. A total of 970 patients were entered into Study NV14256 at forty nine clinical sites. The study had the following three treatment arms (patients): ddC (325), Saquinavir (327), and Saquinavir + ddC (318), The demographic and baseline disease characteristics of the patients in Study NV14256 represented a diverse population of patients with advanced HIV-1 infection and with a wide range of prior antiretroviral treatment regimes.

The utility of the AMPLICOR HIV-1 MONITOR Test to measure the effects of drug treatment over time was evaluated by analyzing the median change from baseline and the DAVGt (difference averaged over time -mean change from baseline over t weeks) in the HIV-1 RNA level, 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 median change from baseline was analyzed over time for each treatment arm of the study, Figure 14 shows the median change from baseline over a 48 week time period for each treatment arm in Study NV14256. Measurable and sustained decreases in HIV-1 RNA levels as determined by the AMPLICOR HIV-1 MONITOR Test were seen. The largest and the most sustained median change in HIV-1 RNA levels at each time point was seen in patients in the combination treatment arm (SAO+ddC).

The utility of serial HIV-1 RNA measurements to assess viral response to antiretroviral treatment was also examined using the Cox Proportional Hazards Model, In this analysis, the three treatment groups were combined and a Cox model, stratified by treatment group, was fit using the following covariates as linear terms: 10910 (baseline HIV-1 RNA), 10910 (last HIV-1 RNA), baseline CD4+ and last CD4+. The hazard ratio in this model was defined as a 10-fold increase in HIV-1 RNA or a decrease of 100 in CD4+ count. As shown in Table 14, the coefficient of the log10 (last HIV-1 RNA) term for this model is statistically significant and positive through Week 40, Accordingly, the HIV-1 RNA Hazard Ratios through Week 40 indicate that the HIV-1 RNA levels et each time point have statistically significant and continuous prognostic value.

In a related analysis of the data, the association between HIV-1 RNA levels and survival time was assessed. For this analysis, survival time was defined as time on study in which a patient survived free of an AIDS Defining Event (ADE) or death. The analyses were performed by constructing Kaplan-Meier Survival Curves for each treatment arm by dividing the patient population within the treatment arm into thirds based on HIV-1 RNA levels, nominally the Lower, Middle, and Upper thirds. The survival curves were plotted as a function of the percent ADE Survival for each patient group (Lower, Middle and Upper) for the number of weeks through Week 8. The Kaplan Meier estimated survival analyses show that patients with low HIV-1 RNA levels (Lower Third) are more likely to survive ADE-free for a longer period of time than patients with high HIV-1 RNA levels, The Kaplan-Meier survival curves for each study treatment arm are shown in Figures 15,16 and 17.

In a separate analysis of the data from Study NV14256, a patient's risk of ADE or death was assessed as a function of the last (most recent) HIV-1 RNA level at various study weeks. In particular, the impact of the last HIV-1 RNA measurement on the hazard for the development of a first ADE or death (based upon clinical events seen later in the study) was assessed. The analysis was performed using a Cox model survival analysis using the covariates log10 (baseline HIV-1 RNA), IOg10 (last HIV-1 RNA), baseline CD4+ and last CD4+. The model was a linear function of the covariates predicting the log of the hazard (ratio) for an ADE. The linear function was then used to determine a Risk Index for all patients in the study. Patients who survived ADE free through Week 4 of the study were sorted by their Risk Index with the first 25% of the patients forming the "low risk" group and the remaining patients equally divided into six separate groups according to their Risk Index. Each of these six groups was then compared to the low risk group using

a survival model and the mean HIV-1 RNA level of each group was plotted versus an ADE hazard ratio calculated using a piecewise exponential model. Figures 18 and 19 show the Hazard Ratios for the time to first ADE versus the 10910 (last HIV-1 RNA) for patients surviving ADE free through Week 4 and patients surviving ADE free through Week 16, respectively. These data show that a patient in this study at Week 4 with an HIV-1 RNA level of 100,000 copies/mL (10910 = 5.0) was 6 times more likely to develop an ADE or to die than was patient with an HIV-1 RNA level of 8000 copies/mL. A patient in this study at Week 16 with an HIV-1 RNA level of 1,000,000 (log10 = 6.0) was 20 times more likely to develop an ADE or to die than was a patient with an HIV-1 RNA level of 8,000 copies/mL.

The data presented here regarding the clinical utility and the use of the AMPLICOR HIV-1 MONITOR Test to monitor the effects of antiretroviral therapy were derived from a single clinical study that compared two drugs and three therapeutic regimens for a specific patient group, Because a single clinical study may not adequately demonstrate the clinical utility of quantitative HIV-1 RNA testing in all patient populations, in all clinical situations, or with all antiretroviral therapies, sufficient care should be taken before extending the interpretation of the data from the study presented here to any individual patient case. As with any diagnostic test, results from the AMPLICOR HIV-1 MONITOR Test should be interpreted with consideration of all relevant clinical and laboratory findings for each patient.

Figure 14
HIV-1 RNA Medians of Change from Baseline Over 48 Weeks
Study NV14256

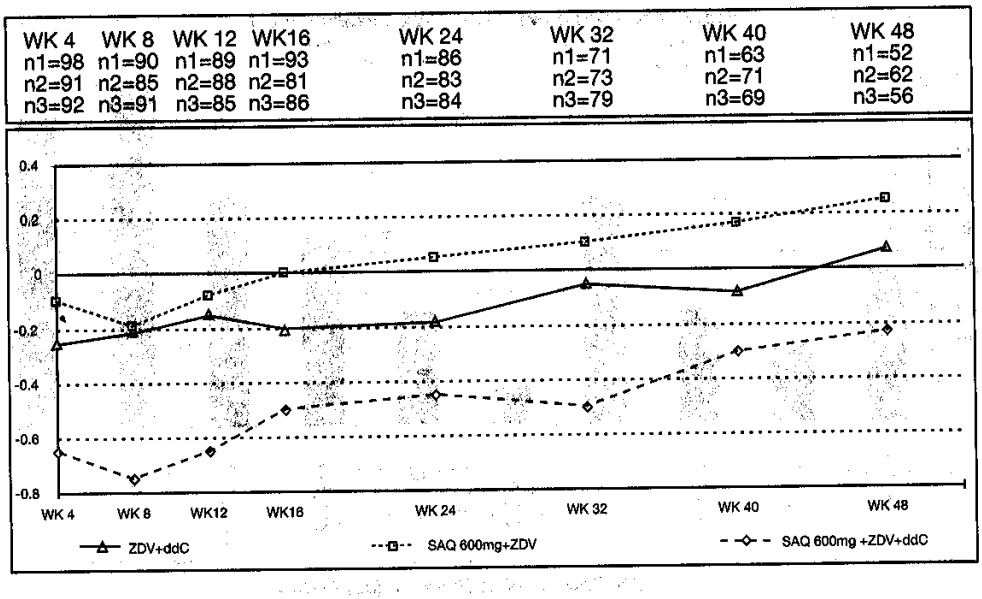


Table 14 *ADE Risk versus Log,o (Last RNA) and Other Covariate Based* on *Time from the Given Study Week for Patients Surviving Through that Week Summary of Cox Model Results -Model Stratified by Treatment Group Study NV14256*

Study Week	Variable	Cox Model Coefficient	Hazard Ratio	p Value
	Log_{10} (Baseline RNA)	0.798	2.22	0.0012
4	Log_{10} (last ANA)	0.4197	1.52	0.0219
	Baseline CD4	-0.0009	0.91	0.5806
	Last CD4	-0.0040	0.67	0.0084
	Log_{10} (Baseline RNA)	0.8096	2.25	0.0004
8	Log_{10} (last ANA)	0.4186	1.52	0.0138
	Baseline CD4	-0.0019	0.83	0.2107

	Last CD4	-0.0049	0.62	0.0006
	Log_{10} (Baseline RNA)	0.8714	2.39	0.0050
16	Log_{10} (last ANA)	0.5385	1.71	0.0046
	Baseline CD4	-0.0015	0.87	0.3510
	Last CD4	-0.0049	0.62	0.0010
	Log_{10} (Baseline RNA)	0.6910	2.00	0.0136
24	Log_{10} (last ANA)	0.7135	2.04	0.0017
	Baseline CD4	-0.0003	0.97	0.8667
	Last CD4	-0.0064	0.53	0.0001
	Log_{10} (Baseline RNA)	0.5926	1.81	0.0492
32	Log_{10} (last ANA)	0.9000	2.46	0.0003
	Baseline CD4	-0.0040	0.67	0.0252
	Last CD4	-0.0023	0.79	0.1523
	Log_{10} (Baseline RNA)	0.8003	2.23	0.0159
40	Log_{10} (last ANA)	0.7086	2.03	0.0096
	Baseline CD4	-0.0030	0.74	0.1282
	Last CD4	-0.0031	0.73	0.0955
	Log_{10} (Baseline RNA)	0.5993	1.82	0.0978
48	Log_{10} (last ANA)	0.5631	1.76	0.0629
	Baseline CD4	-0.0011	0.89	0.6168
	Last CD4	-0.0079	0.45	0.0016
	Log_{10} (Baseline RNA)	1.0917	2.98	0.0127
56	Log_{10} (last ANA)	0.0835	1.09	0.8025
	Baseline CD4	-0.0022	0.80	0.4560
	Last CD4	-0.0072	0.49	0.0201
	Log_{10} (Baseline RNA)	1.2803	3.60	0.0135
64	Log_{10} (last ANA)	-0.2500	0.78	0.4988
	Baseline CD4	-0.0006	1.07	0.8531
	Last CD4	-0.0085	0.43	0.0213

' *Hazard Ratio due* to a *10-fold increase* in *HIV-1 RNA or* a *decrease of* 100 in *CD4+ count.*

Figure 15
Kaplan Meier Estimated Survivals – Study NV14256
Treatment Group: SAD 600 mg
Patients Surviving ADE Free Through Week 8

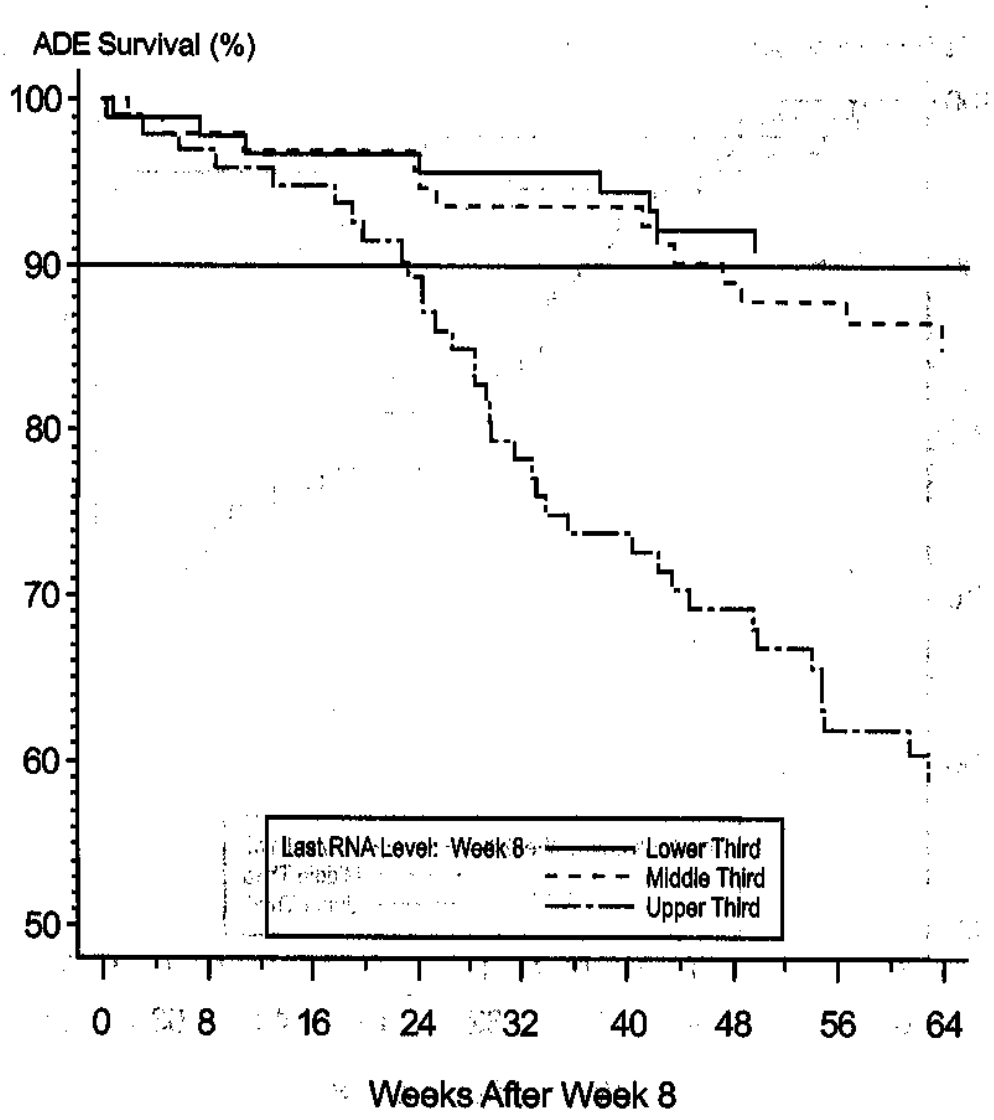
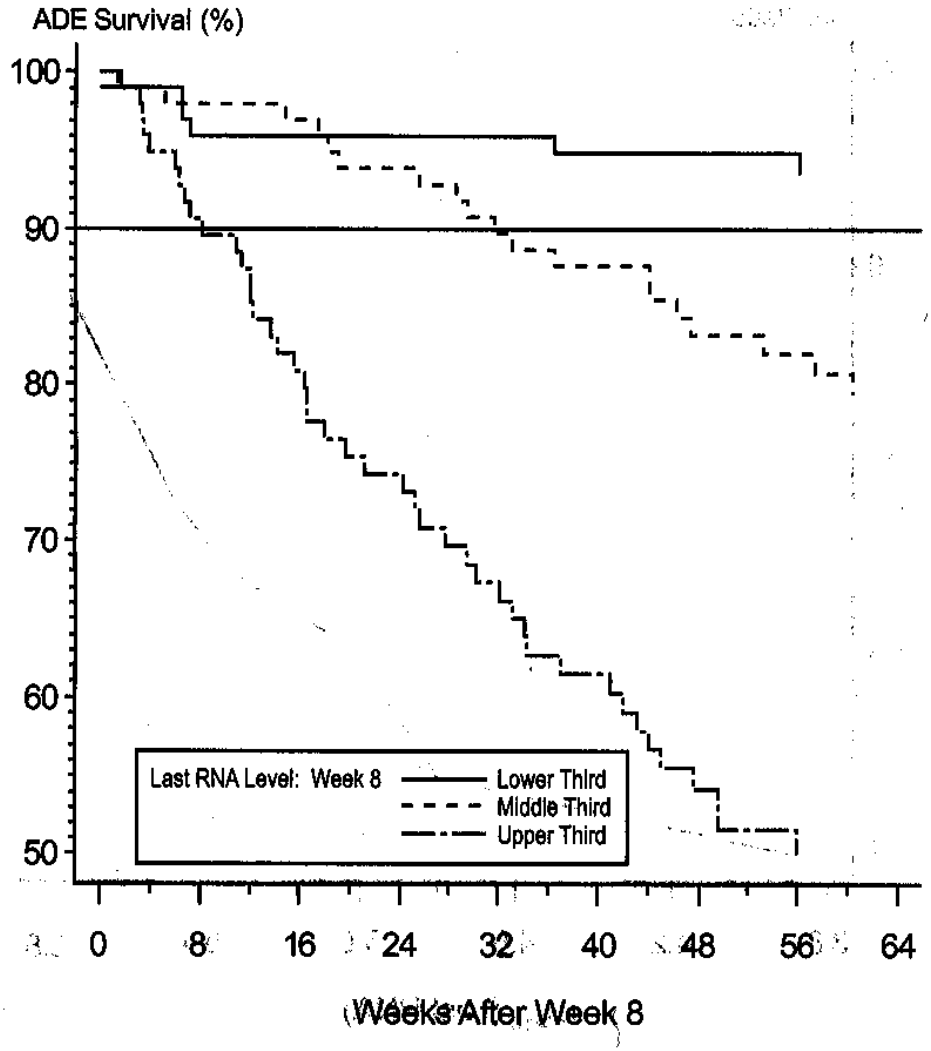


Figure 16
Kaplan Meier Estimated Survivals – Study NV14256
Treatment Group: ddC
Patients Surviving ADE Free Through Week 8



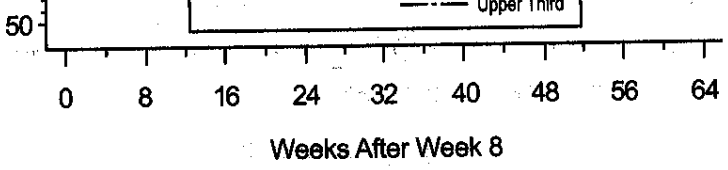


Figure 18
Hazard Ratio for Time to First ADE
Patients Surviving ADE Free Through Week 4
Study NV14256

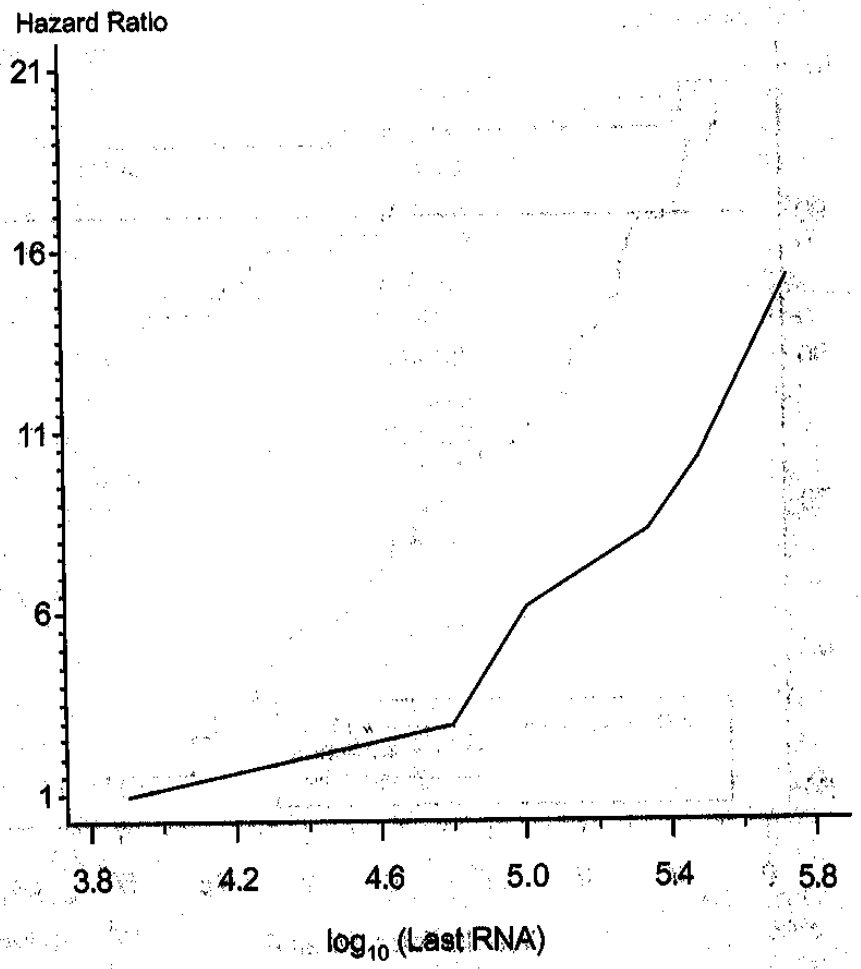
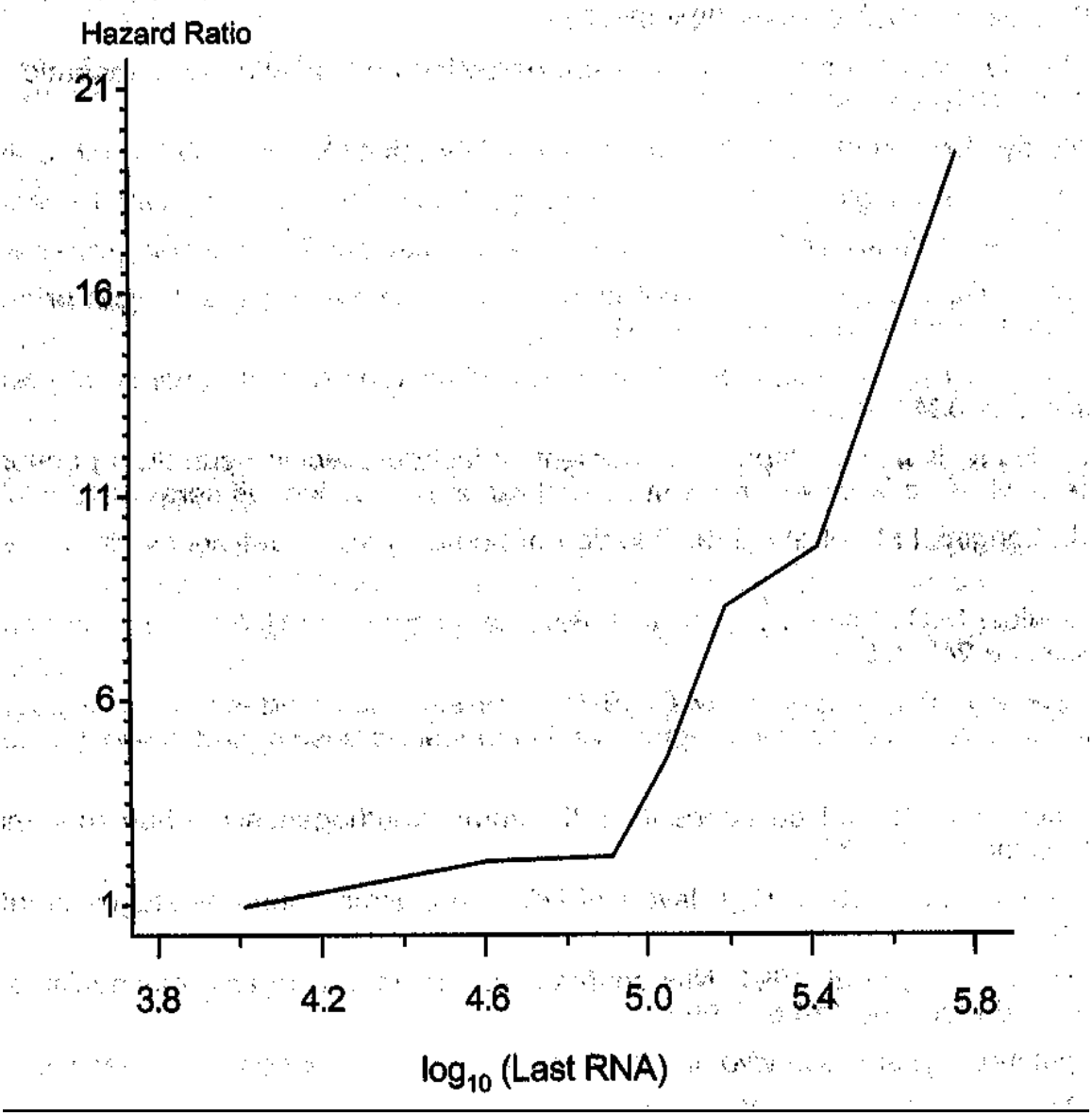


Figure 19
Hazard Ratio for Time to First ADE
Patients Surviving ADE Free Through Week 16
Study NV14256



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

Prepared by: ACTG Laboratory Technologist Committee

Preparation Date: 01 June 2004

Date Implemented into the Laboratory: _____

Updated on:

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Supersedes Archived Protocol: DAIDS Virology Manual for HIV Laboratories, Version January 1997