NucliSens® HIV-1 QT

NASBA Diagnostics

For *in vitro* diagnostic use.

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NucliSens® HIV-1 QT

For *in vitro* diagnostic use.

1. Intended use

The NucliSens® HIV-1 QT 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 176 to $3.47X10^6$ copies/mL.

The test is intended for use in conjunction with clinical presentation and other laboratory markers of disease progression for prognostic assessment of HIV-1 infected patients, and for monitoring the effects of anti-retroviral therapy by serial measurements of plasma HIV-1 RNA for pediatric and adult patients with baseline viral loads greater than 93,000 and 28,000 copies of HIV-1 viral RNA/mL respectively.

The NucliSens® HIV-1 QT assay is not intended to be used as a screening test for HIV-1 nor is it to be used as a diagnostic test to confirm the presence of HIV-1 infection.

2. Summary and explanation of the test

Acquired Immunodeficiency Syndrome (AIDS) is an immunosuppressive disorder characterized by depletion of the $CD4^+$ T-cell population. A progressive, severe immunodeficient state is accompanied by a broad variety of clinical manifestations including opportunistic infections, an array of malignancies, and the frequent presence of neurological disorders.

Human Immunodeficiency Virus (HIV) is the etiologic agent of AIDS and can be transmitted by sexual contact, through contaminated injection needles or through administration of contaminated blood or blood products. HIV is also capable of passing through the placenta.

So far, two types of HIV have been found to cause AIDS: HIV-1, first isolated in 1983, and HIV-2, a second distinct but related type, first isolated in 1985.

The conventional method for detection of HIV infection is through serologic identification of an immunologic response to HIV; e.g., by means of enzyme-linked immunosorbent assays (ELISA), and confirmation of the results with more specific assays, such as Western blot.

Unlike these indirect methods, nucleic acid amplification techniques such as Reverse Transcription PCR (RT-PCR) and Nucleic Acid Sequence Based Amplification (NASBA)[1,2,4] do not depend on the development of an immunologic response (i.e. the formation of antibodies) to HIV, which normally takes several weeks to appear after an infection.[5] These nucleic acid based methods directly test for HIV RNA. In addition, they are more sensitive than p24 antigen assays.[4, 6-9] The advantage of NASBA over RT-PCR is that it requires no thermocycling since the amplification for NASBA occurs at a constant temperature.[4]

Nucleic acid amplification is suitable for the quantitation of HIV-1 RNA in human plasma samples,[2,3,8] which is referred to as “viral load”. HIV-1 RNA viral load appears to be a valuable marker for the prediction of disease progression and for monitoring the efficacy of anti-viral therapy.[8,10,11]

Quantitation with NucliSens® HIV-1 QT is based on co-amplification of HIV-1 RNA present in the sample together with three RNA internal calibrators.[12] This technique has proven to be better than certain other quantitation methods in several aspects.[13,14] The quantity of amplified RNA is measured by means of electrochemiluminescence (ECL).[15,16]

3. Principle of the procedure

The NucliSens® HIV-1 QT assay comprises five separate stages.

a. Nucleic acid release
b. Nucleic acid isolation
c. Nucleic acid amplification
d. Nucleic acid detection
e. Quantitation of HIV-1 RNA

3.1. Nucleic Acid Release

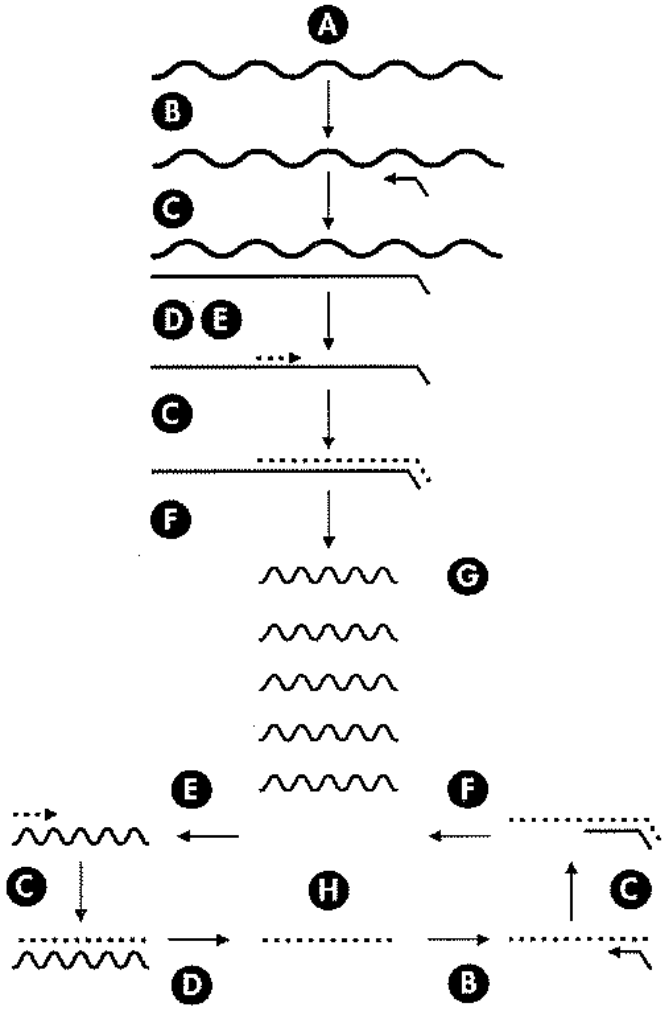
The sample is added to NucliSens® Lysis Buffer containing guanidine thiocyanate and Triton X-100. Any viral particles and cells present in the sample are disintegrated; any RNases and DNases present in the sample are inactivated. Nucleic acid is released.

3.2. Nucleic Acid Isolation

Three synthetic RNAs (Qa, Qb, Qc) of known high, medium and low concentration, respectively, are added to the Lysis Buffer containing the released nucleic acid. These RNAs serve as internal calibrators, each differing from the HIV-1 wild-type (WT) RNA by only a small sequence. Under high salt conditions, all nucleic acid in the buffer, including the calibrators, binds to silicon dioxide particles.[3] These particles, acting as the solid phase, are washed several times. Finally, the nucleic acid is eluted from the solid phase.

3.3. Nucleic Acid Amplification (see Figure 3-1 for illustration)

WT HIV-1 RNA present in the eluted nucleic acid is co-amplified along with the three internal calibrators. As illustrated in figure 1, amplification is based on repeated cycles of transcription reactions. Multiple copies of each WT and calibrator RNA target sequence are synthesized by T7-RNA polymerase by means of an intermediate DNA molecule that contains a double-stranded T7-RNA polymerase promoter. Each transcribed RNA molecule enters a new amplification cycle. The DNA intermediate is generated through a process that involves the binding of a primer to the RNA template, the extension of primer by AMV-RT (Avian Myeloblastosis Virus Reverse Transcriptase) to form an RNA-DNA duplex, the degradation of the RNA strand of the duplex by RNase H, the binding of a second primer to the remaining DNA strand and, finally, the extension of the second primer to form the double-stranded T7-RNA polymerase promoter needed for transcription. Once transcription is initiated, the RNA transcripts which are 'negatives' of the original RNA present in the sample will be subject to the same process, only in this case extension is not restricted to the second primer, since the extension product of the first primer will also be extended. The primers (one of which contains the sequence of the T7-RNA polymerase promoter) are complementary to two different parts of the HIV-1 RNA. This pair of primers defines, and allows the amplification of, a sequence within the HIV-1 gag region. Since the Nucleic Acid Sequence-Based Amplification (NASBA) process requires no strand separation, amplification is isothermal and continuous



Legend

- A ss RNA (sense)
- B Primer 1
- C AMV-RT
- D RNase H
- E Primer 2
- F T7 RNA pol
- G ss RNA (antisense) amplificate
- H Isothermal amplification

- sense RNA
- Primer 1
- Primer 2
- antisense DNA
- sense DNA
- antisense RNA

Figure 3-1: Nucleic acid amplification principle

3.4. Nucleic Acid Detection

The quantitation of HIV-1 RNA in a sample is based on measurement of electrochemiluminescence (ECL) with the NucliSens® Reader. To differentiate between the amplicons (WT, Qa, Qb and Qc), aliquots of the amplified sample are added to four hybridization solutions, each specific for one type of amplicon. Here, the respective amplicons are hybridized with a bead-oligo (i.e. an oligo bound to streptavidin coated paramagnetic beads acting as the solid phase) and a ruthenium-labeled probe. The paramagnetic beads carrying the hybridized amplicon/probe complex are captured on the surface of an electrode by means of a magnet. Voltage applied to this electrode triggers the (ECL) reaction. The light emitted by the hybridized ruthenium-labeled probes is proportional to the amount of amplicons, which in turn are proportional to the HIV-RNA in the input samples. Calculation based on the relative amounts of the four amplicons gives an estimation of the amount of HIV-1 RNA in the sample.

3.5. Quantitation of HIV-1 RNA

The NucliSens® HIV-1 QT system uses three internal calibrators for quantitation. These internal calibrator RNAs are produced *in vitro* from plasmid constructs encoding the wild-type *gag* region sequence. These three calibrator RNAs are identical to the wild-type sequence except for a 20 base sequence recognized specifically by the detector probe. In the case of calibrator RNAs (designated Qa, Qb, and Qc), the detector probe site was mutagenized to generate three distinct sequences, but similar in A, C, G, and T content to the wild type sequence. Thus, the calibrator RNAs are amplified with the same kinetics as the wild-type target, each reaction product is captured onto the magnetic bead with the same efficiency, and each calibrator reaction product can be distinguished from the other and from the wild-type product by the detector probes with distinct sequences. Because the A, C, G, and T content in each detector site is maintained, the melting temperature of all four probes is similar.

The internal calibrators for quantitation are introduced into the specimen immediately after the initial lysis step. Importantly, the three calibrators are included at known copy numbers (Qa ~ 10^5, Qb ~ $10^{4.3}$, Qc ~ $10^{3.6}$). These calibrators are co-extracted and co-amplified with the wild-type nucleic acid in a single tube. The reaction product is then divided into four independent detection assays, each with one of the calibrator detector probes and one with the wild-type detector probe. At the completion of the hybridization reactions, the four products are loaded into the NucliSens® Reader. The ECL signal for each of the hybridization reactions is determined and the input copy number for wild-type HIV-1 RNA, relative to the input quantities of calibrator RNAs is determined by calculating the ratio of ECL signals for WT to Qa, Qb, and Qc.

The actual computation of the input quantity of wild-type RNA is achieved through the application of a specific curve-fitting program.[12] Importantly, the inclusion of the calibrators in the analysis of the independent samples permits individual determinations of the acceptance of each wild-type calculation through a validated algorithm designed to evaluate the relationship of the calibrator signals to each other. The three calibrator signals (Qa, Qb, Qc) are corrected for background noise and checked against a number of parameters: upper detection limit, fixed minimum values, variance, and correlation. If the Qc calibrator has been discarded as a result of the check, the remaining two calibrators (Qa and Qb) are used to calculate the wild-type RNA concentration.

4. Reagents

For *in vitro* diagnostic use

Availability

NucliSens® Lysis Buffer (9 ml/tube)	Product Number 84047
NucliSens® Isolation reagents	Product Number 84160
NucliSens® HIV-1 QT Amplification reagents	Product Number 84152
NucliSens® HIV-1 QT Detection reagents	Product Number 84043
NucliSens® HIV-1 RNA Controls	Product Number 84010

For technical assistance, contact bioMeriéux, Inc. at 1-800-682-2666.

4.1. Warnings and Precautions

For *in vitro* diagnostic use.

- **Warning**: As no test method can offer complete assurance that infectious agents are absent, all materials of human origin should be handled as though they contain potentially infectious agents.
- **Warning**: Lysis Buffer and Wash Buffer contain guanidine thiocyanate. Guanidine thiocyanate is harmful if inhaled, or comes in contact with skin or if swallowed. Contact with acid liberates very toxic gas.
- The waste material from the extraction process should be disposed of separately and not mixed with any other laboratory waste.
- Prepare Lysis Buffer **before** starting nucleic acid release. Make sure any crystals in the Lysis Buffer have dissolved.
- Make sure Lysis Buffer and samples are at room temperature (15-30°C) before starting nucleic acid release.
- Specimens that have been repeatedly frozen and thawed or that contain particulate matter may yield erroneous results. However, specimens containing particulate matter may be used for testing after centrifugation.
- Label tubes with appropriate patient information.
- Do not use Lysis Buffer after the expiration date given on the package label.
- Return unopened Lysis Buffer tubes to 2-8°C as soon as possible.
- Do not uncap tubes in the presence of other open tubes containing patient material.
- Mix Lysis Buffer and samples thoroughly before use.
- Keep testing areas separate from areas where blood or blood products are stored.
- Do not pipette any of the materials by mouth. Do not smoke, eat or drink in areas in which specimens or kit reagents are handled.
- Use disposable powder free gloves and handle all materials used in the test including samples, reagents, pipettes, etc., cautiously as though capable of transmitting infectious agents. Consult a physician immediately in the event that contaminated materials are ingested or come in contact with open lacerations, lesions or other breaks in the skin.
- Immediately clean up any spillage containing potentially infectious agents with a 1:10 dilution of 5% (w/v) (freshly prepared) sodium hypochlorite. Dispose of the cleaning material by an accepted method. Rinse well with distilled water to remove residual bleach.
- Do not perform the test in the presence of reactive vapors (e.g., from sodium hypochlorite, acids, alkalis or aldehydes) or dust because the enzymatic activities may be affected.
- Dispose of all specimens and materials used to perform the test as if they contain infectious agents.
- **Avoid contamination:**
  Perform nucleic acid release, isolation, amplification and detection in separate laboratory areas.
  - Perform step 1 to 4 (Section 6.3) of amplification in a fume hood; step 6 should preferably also be performed in a fume hood.
  - Air from the detection area must not be allowed to enter other areas (detection should preferably be performed in a fume hood).
  - Keep all tubes and vials closed when not in use.
  - Do not use pipettes and other equipment that have been used in one laboratory area in other areas.
  - Use a fresh pipette or pipette tip for each pipetting action.
  - Use pipettes with aerosol resistant tips or air displacement pipettes for fluids possibly containing nucleic acid.
  - Pipetting of solutions must be performed out of or into an isolated tube that is opened and closed exclusively for this action. All other tubes and vials should be kept closed and separated from the one being handled.

  - Use disposable powder free gloves when working with clinical material possibly containing WT-RNA or amplified material. If possible, change gloves after each pipetting step in the test procedure, especially after contact with possibly contaminated material.
  - With air displacement pipettes, aerosol formation can be decreased by pipetting until the first stop of the pipette.
  - Collect used disposable material in a container. Close and remove container after each test run.
  - Soak tube racks used during nucleic acid isolation in an alkaline detergent for at least one hour after each test run.
  - Clean the centrifuge rotor used for nucleic acid isolation with an alkaline detergent directly after use; then rinse with water and dry.
- Make sure heating blocks and water baths used in the amplification lab area and the temperature inside the blocks is within the range given in the instructions under Section 6.3 “Nucleic Acid Amplification.” Use a calibrated thermometer to measure the temperature. Actual temperatures exceeding 41.5°C may cause damage to the enzymes and impede amplification.
- Do not use materials after the expiration date given on the package label. Return unopened amplification reagents and detection reagents immediately to 2-8°C. Return unopened isolation reagents to 2-30°C.
- Prepare reagents prior to starting nucleic acid release, isolation, amplification and detection, respectively.
- Make sure reagents and samples are at room temperature (15-30°C) before starting nucleic acid release, isolation, amplification and detection, respectively.
- All reagents must be mixed thoroughly before use. **Note: Enzymes must not be vortexed.** Ensure complete reconstitution of the lyophilized sphere enzymes by gently flicking the closed tube with your finger after addition of Enzyme Diluent (refer to 6.3 “Nucleic Acid Amplification”).
- Before opening a tube that contains lyophilized material, make sure the lyophilized material is at the bottom of the tube.
- Storage of prepared or opened isolation and amplification reagents is not recommended. Use immediately.
- When performing a NucliSens® HIV-1 QT test, the first six digits of each of the Amplification and Detection reagent modules lot numbers printed on the box label should match. The Lysis Buffer and Isolation module used in a test run may be from any lot.
- Work flow in the laboratory is an important consideration in any quality control program. Nucleic acid release, isolation, amplification, and detection are performed in separate areas to aid in preventing contamination. The work should flow in one direction only from release, to isolation, amplification, and finally to detection. Once a technician has moved to the next area, he or she should not return to a previous area during that day. All supplies for a particular procedure should be stored in the area where that procedure is performed and should not be moved between areas. Gloves should be removed and disposed of before leaving one area to proceed to the next. Lab coats should be specific to an area and never worn outside of that area.

4.2. NucliSensÒ HIV-1 QT reagents for 50 tests

4.2.1. Nucleic Acid Release

Storage

Store NucliSens® Lysis Buffer at 2-8°C in an area intended for the preparation of specimens for nucleic acid release. Remove reagents from the refrigerator only in quantities needed for the number of tests to be performed. Protect from excessive heat or light.

Contents

Each Lysis Buffer module contains reagents for 50 nucleic acid releases.

50 tubes **Lysis Buffer** (9 ml/tube) 5 mol/l guanidine thiocyanate, Triton X-100, TRIS/HCl.

R20/21/22: Guanidine thiocyanate is harmful by inhalation, in contact with skin and if swallowed.
R32: Contact with acid liberates very toxic gas.

Harmful

4.2.2. Nucleic Acid Isolation

Storage

Store reagents supplied in this kit at 2-30°C in the dedicated isolation laboratory area. Remove reagents from the refrigerator only in quantities needed for the number of tests to be performed. Protect from excessive heat or light when in use. The expiration date printed on the kit indicates the date beyond which reagents should not be used.

Discard any unused reconstituted reagents. Storage of prepared or opened Isolation reagents is not recommended. Use immediately.

Contents

NucliSens® Isolation reagents (for 50 tests)

5 tubes (22 ml/tube) **Wash Buffer**
5 mol/l guanidine thiocyanate, TRIS/HCl.

R20/21/22: Guanidine thiocyanate is harmful by inhalation, in contact with skin and if swallowed.
R32: Contact with acid liberates very toxic gas.

Harmful

5 tubes (0.8 ml/tube) **Silica**
Hydrochloric acid-activated silicon dioxide particles

5 tubes (1.5 ml/tube) **Elution Buffer**
TRIS/HCl
Color code: white capped tube.

5 x 6 mg **Calibrators**
Lyophilized synthetic RNA (Qa, Qb and Qc) sphere; each tube contained in a foil pack with silica gel desiccant. Color code: yellow capped tube.

4.2.3. Nucleic Acid Amplification

Storage

Store at 28°C in the dedicated amplification laboratory area. Remove reagents from the refrigerator only in quantities needed for the number of tests to be performed. Protect from excessive heat or light. The expiration date printed on the kit indicates the date beyond which reagents should not be used. Storage of opened or prepared Amplification reagents is not recommended. Use immediately. Discard any unused reconstituted reagents.

Contents

NucliSens® HIV-1 QT Amplification reagents for 50 tests.

5 x 6.5 mg **Enzymes**
Lyophilized sphere AMV-RT, RNase H, T7-RNA polymerase and BSA; each tube contained in a foil pack with silica gel desiccant. Color code: red capped tube.

5 x 0.5 ml **Enzyme Diluent**
TRIS/HCl. Color code: red capped tube.

5 x 10 mg **Primers**
Lyophilized sphere with synthetic primers, nucleotides, and stabilizers; each tube contained in a foil pack with silica gel desiccant. Color code: blue capped tube.

5 x 0.5 ml **Primer Diluent**
TRIS/HCl, 30% DMSO. Color code: blue capped tube.

4.2.4. Nucleic Acid Detection

Storage

Store at 2-8°C in the dedicated detection laboratory area. Remove reagents from the refrigerator only in quantities needed for the number of tests to be performed. Protect from excessive heat or light. The expiration date printed on the kit indicates the date beyond which reagents should not be used.

Contents

NucliSens® HIV-1 QT Detection reagents for 50 tests

2 x 1.68 ml **Bead-oligo**
DNA oligo bound to streptavidin-coated paramagnetic beads with preservative. Color code: pink capped tube.

1 x 0.84 ml **WT Probe**
Ruthenium-labeled DNA oligo with preservative. Color code: white capped tube.

1 x 0.84 ml **Qa Probe**
Ruthenium-labeled DNA oligo with preservative. Color code: red capped tube.

1 x 0.84 ml **Qb Probe**
Ruthenium-labeled DNA oligo with preservative. Color code: yellow capped tube.

1 x 0.84 ml **Qc Probe**
Ruthenium-labeled DNA oligo with preservative. Color code: blue capped tube.

2 x 15 ml **Detection Diluent**
TRIS/HCl with preservative.

1 x 1.7 ml **Instrument Reference Solution**
Streptavidin-coated paramagnetic beads.

4.3. Additional materials required (but not supplied)

4.3.1. Nucleic Acid Release

- Tabletop centrifuge.
- Tubes with cap for specimen storage.
- Calibrated micropipettes with variable settings for 10 through 1,000 µl delivery volumes.
- Sterile-packaged, disposable, aerosol-resistant tips.
- Absorbent tissue.
- Disposable powder free gloves.

4.3.2. Nucleic Acid Isolation

- 70% to 72% (v/v) Ethanol prepared from 200-proof ethanol; use nuclease-free (not DEPC-treated) water for dilution.
- Acetone (ACS quality).
- RNase free 1.5ml test tubes (preferably screw-cap), which can be centrifuged at 10,000 g, and 1.5 ml test tube racks.
- Calibrated disposable tip pipettes, volume range 5 to 1,000 µl **(use two different pipettes for removing supernatant and adding wash fluid)** with aerosol resistant tips.
- Heating block, capable of heating 1.5 ml test tubes to 56 ± 1°C.
- Timer.
- Vortex.
- Table top centrifuge for 9 ml Lysis Buffer tubes (capable of 1,500 g).
- Table top centrifuge for 1.5 ml test tubes (capable of 10,000 g).
- Waste container with cap.
- Large disposable transfer pipettes or disposable 10 ml tubes with screw-cap for removal of supernatant from 9 ml Lysis Buffer tubes (refer to 6.2 “Nucleic Acid Isolation”).
- Tube racks suitable for 9 ml Lysis Buffer tubes.

4.3.3. Nucleic Acid Amplification

- Calibrated disposable tip pipettes (volume range 5 to 200 µl) with aerosol resistant tips.
- Two heating blocks capable of heating 1.5 ml test tubes to 41 ± 0.5°C and 65 ± 1°C.
- Two calibrated thermometers (precision ± 0.2°C).
- Circulating water bath capable of heating 1.5 ml test tubes to 41 ± 0.5°C, with (floating) test tube holder.
- Timer.
- Vortex.
- Table top centrifuge for 1.5 ml test tubes (capable of 10,000 g).
- 1.5 ml test tube racks.
- Waste container with cap.

4.3.4. Nucleic Acid Detection

- RNase free 1.5 ml test tubes (preferably screw-cap), which can be centrifuged at 10,000 g, and 1.5 ml tube racks.
- Calibrated disposable tip pipettes (volume range 5 to 1,000 µl) with aerosol resistant tips.
- Water bath capable of heating 5 ml tubes to 41 ± 0.5°C.
- Timer.
- Vortex or Shaker capable of 1,100 rpm.

- Table top centrifuge for 1.5 ml test tubes (capable of 10,000 g).
- NucliSens® Reader with Personal Computer, Assay Buffer and Cleaning Solution.
- Polypropylene tubes (5 ml) for hybridization (250 tubes per kit) and tube rack (rack is supplied with the NucliSens® Reader).
- Repeating pipette with disposable tips (0.50 ml and 1.25 ml).
- Adhesive tape.
- Waste container with cap.

4.4. Reagent Preparation

4.4.1. Nucleic Acid Release

Pre-warm Lysis Buffer tubes for about 30 minutes at 37°C to make sure any crystals in the Lysis Buffer have dissolved. Cool down to room temperature (15-30°C).

4.4.2. Nucleic Acid Isolation

Wash Buffer

- Pre-warm a Wash Buffer vial for about 30 minutes at 37°C before starting the assay to make sure that any crystals in the Wash Buffer have dissolved (invert regularly).
- Cool down to room temperature (15-30°C).
- Protect the Wash Buffer from excessive light.

Silica

- Vortex the tube before starting the isolation procedure until an opaque suspension is formed. The Silica particles will settle again loosely.
- Vortex again **before each** pipetting step.

Calibrator Solution

- Reconstitute the Calibrators (yellow) in 220 µl Elution Buffer (white).
- Vortex the solution and spin tubes at 10,000g for 15 seconds.
- **Note:** Use within 1 hour after preparation.

4.4.3. Nucleic Acid Amplification

Primer solution

- Add 120 µl Primer Diluent (blue capped tube) to the lyophilized primers (blue capped tube), using a 200 µl pipette.
  Note: Ensure that Primer Diluent is at room temperature before use.
- Immediately after addition of Primer Diluent, vortex well until clear. **Do not spin!**
  Note: Use within 1 hour after preparation.

Enzyme solution

- Add 55 µl Enzyme Diluent (red capped tube) to the lyophilized Enzymes (red capped tube).
- Allow to stand for 15 minutes; ensure complete reconstitution of the lyophilized sphere enzymes by gently flicking the closed tube with your finger after addition of Enzyme Diluent. **Do not vortex!** Spin before use at 10,000 g for 15 seconds.
- **Note:** Use within 1 hour after preparation.

4.4.4. Nucleic Acid Detection

Hybridization solutions 1 to 4 (for hybridization of WT, Qa, Qb and Qc, respectively)

- Hybridization solution 1
  -Vortex Bead-oligo (pink capped tube) until an opaque solution is formed. Immediately after vortexing:
  -Add 130 µl Bead-oligo to a fresh test tube.
  -Add 130 µl WT Probe (white capped tube) to that tube.
- For hybridization solutions 2 to 4 follow the same procedure, replacing the WT Probe by Qa (red capped tube), Qb (yellow capped tube) and Qc (blue capped tube) Probe, respectively.

- Vortex hybridization solutions before use.
- **Note:** Use within 1 hour after preparation.

4.5. Indications of Instability

Alterations in the physical appearance of test kit materials may indicate instability or deterioration. Expiration dates shown on component labels indicate the date beyond which components should not be used.

4.6. Instruments

For any instruments used, review the manual provided by the manufacturer for additional information regarding the following:

- Installation and special requirements.
- Operation principles, instructions, precautions and hazards.
- Manufacturer's specifications and performance capabilities.
- Service and maintenance information.
- Quality control information

5. Specimen collection, storage and transport

Warning: Handle all specimens as if capable of transmitting infectious agents. However, as no test method can offer complete assurance that infectious agents are absent, all materials of human origin should be handled as though they contain potentially infectious agents. Handle specimens and materials contacting specimens as if potentially infectious biological materials in accordance with "Universal Precautions for Prevention of Transmission of Human Immunodeficiency Virus, Hepatitis B Virus, and other Bloodborne Pathogens in Health-Care Settings" (CDC, MMWR, June 24, 1988).

Note: Lysis Buffer will inactivate up to 5.3 log 10 of the HIV-1 virus.

5.1. Specimen collection

Plasma should be used as the sample input. The standard sample volume is 1.0 ml (in the 9.0 ml Lysis Buffer tube).

Blood should be collected in sterile tubes using EDTA, citrate or heparin as an anticoagulant. Clinical results indicate that there is no significant difference in the quantitation of HIV-1 RNA with the NucliSens® HIV-1 QT system for these three anticoagulants.

- Whole blood specimens collected using EDTA as an anticoagulant can be stored at room temperature (15-30°C) for up to 24 hours before processing without any loss of RNA. EDTA plasma can be stored at 2-8º C for up to 14 days.
- Plasma collected using EDTA, citrate or heparin is stable for $\geq$ 1 year at –70^{o}C and can be frozen and thawed up to three times with no significant loss of HIV-1 RNA reported by the NucliSens® HIV-1 RNA QT system. Specimens repeatedly frozen and thawed or those containing particulate matter may give erroneous results.
- In Lysis Buffer, EDTA plasma specimens can be stored:
  -up to one year at –70°C **or**
  -for a maximum of 14 days at 2-8°C **or**
  -for a maximum of 24 hours at room temperature (15-30°C).

Note: **Do not store specimens in Lysis Buffer at –20°C.**

- Purified RNA eluate (post Isolation) can be stored for 12 months at –20 or –70^{o}C or for 14 days at 2-8^{o}C.
- Amplified material may be stored for 12 months at –20^{o}C.

5.2. Specimen transport

If the shipment can be accomplished within 24 hours of collection, the specimen may be shipped at room temperature (15-30°C). Otherwise, the specimen should be shipped on dry ice. Specimens may be transported to the laboratory via courier, airfreight, or regular mail in accordance with applicable Federal, state and local regulations that apply to the transportation of diagnostic specimens.

Note: Handle tubes stored at –70°C (or on dry ice) with care to avoid breakage associated with low temperature storage.

Note: Specimens in Lysis Buffer should be shipped at or below -30°C (i.e, on dry ice).

6. NucliSensÒ HIV-1 QT procedure

Run Size. The quantities given for preparation of reagents are sufficient for a series of 10 samples. Each NucliSens® HIV-1 QT kit contains reagents sufficient for five 10-test batches. Each batch may be performed in a run separate from or simultaneous with other batches. It is recommended to include a high Positive Control, low Positive Control, and a Negative Control with the first run of each kit lot to verify product performance. The inclusion of a low Positive and a Negative Control with each subsequent run of the same kit lot is also recommended.

6.1. Nucleic Acid Release

1. Centrifuge the Lysis Buffer tubes at 1,500 g for 2 minutes to remove all fluid from the cap. Keep Lysis Buffer tubes upright and handle carefully to avoid splashing fluid back into cap area. Put the Lysis Buffer tubes in one of the racks and place in the hood. **Note**: Before spinning, Lysis Buffer tubes should be warmed at 37°C for 30 minutes. Mix thoroughly and make sure that any crystals have dissolved.
2. Uncap a single sample tube. Without putting the cap down, pipet 1 ml of plasma using an appropriate pipette with aerosol resistant tip. Close the sample tube. Place a single Lysis Buffer tube into a rack designated as a transfer rack. Uncap the single Lysis Buffer tube without putting the cap down. Dispense the sample into the Lysis Buffer tube. Close the Lysis Buffer tube and place it back into the Lysis Buffer tube rack. Discard the pipette tip. **Note**: The use of a transfer rack is recommended for each transfer to individual Lysis Buffer tubes in order to reduce the possibility of cross-contamination.
3. Repeat the step above until all samples are transferred to Lysis Buffer tubes. Use a fresh pipet tip for each transfer and open only one sample tube and one Lysis Buffer tube at a time to avoid cross contamination of samples.
4. Vortex the Lysis Buffer tubes to mix the sample with the Lysis Buffer. After all of the sample additions are completed, allow the Lysis Buffer tubes to incubate for 30 minutes at 37°C.
5. Centrifuge the Lysis Buffer tubes at 1,500 g for 2 minutes to spin all fluid down to the bottom of the tubes before opening. Keep Lysis Buffer tubes upright and handle carefully to avoid splashing fluid back into cap area. Put the Lysis Buffer tubes in one of the racks and place in the hood.

Note: If, after nucleic acid release, the Lysis Buffer tubes with sample are not to be immediately used for isolation, store according to the instructions under Section 5.1 "Specimen collection" in this package insert.

6.2. Nucleic Acid Isolation

1. Spin Lysis Buffer tubes containing sample (i.e. centrifuge at 1,500 g for 2 minutes).

   Note: Before spinning, frozen Lysis Buffer tubes must be thawed at 37°C for about 30 minutes until any crystals have dissolved completely.
2. For each Lysis Buffer tube:

-Add 20 µl of prepared Calibrator Solution.
-Invert.
-Spin Lysis Buffer tubes 2 minutes at 1,500 g.

3. For each Lysis Buffer tube:

   -Vortex the silica suspension.
   -Add 50 µl silica suspension. Invert.

4. Leave Lysis Buffer tubes for 10 ± 1 minutes at room temperature (15-30°C).

   Note: Vortex or invert Lysis Buffer tubes regularly (rotator may be used), e.g. every two minutes, to prevent silica from settling on the bottom.

5. Spin Lysis Buffer tubes for 2 minutes at 1,500 g.

   Note: Use **two** pipettes (1,000 µl with disposable tips), vacuum, or aspirate with transfer pipettes; if using pipettes use the **first** one exclusively for **removing** supernatant (step 6 and 7) and the **second** one exclusively for **adding** wash fluid (step 7).

6. Remove the supernatant from the Lysis Buffer tubes:

   For each 9 ml Lysis Buffer tube:
   Note: method A or method B can be used as an alternative

   A:
   -Remove the supernatant with a fresh disposable large transfer pipette, leaving about 0.5 ml residual fluid in the tube.
   -Discard pipette.
   -Remove the 0.5 ml residual fluid with the first 1,000 µl disposable tip pipette, using a fresh sterile tip; avoid whirling up the pellet.

   B:
   -Carefully decant all supernatant from the Lysis Buffer tube into a fresh disposable 10 ml tube with screw cap.
   -Close and discard the 10 ml tube.

7. Wash the silica pellet in the Lysis Buffer tubes five times:

   -twice **(2 x)** with Wash Buffer;

   Note: After first addition of Wash Buffer, transfer the content of each 9 ml Lysis Buffer tube to a fresh 1.5 ml test tube using the first 1,000 µl disposable tip pipette and a fresh sterile tip for each Lysis Buffer tube. Spin tube and continue wash procedure.

   -twice **(2 x)** with 70% ethanol;
   -once **(1 x)** with acetone.
   -The 1.5 ml test tubes are referred to as test tubes in the following.

Wash procedure:

-Add 1 ml of Wash Buffer to each test tube with the second 1,000 µl disposable tip pipette, using a fresh sterile tip for each test tube.
-Vortex test tubes until the pellets are completely resuspended.
-Spin test tubes 30 seconds at 1,000 g.
-Remove the supernatant with the first 1,000 µl disposable tip pipette, using a fresh sterile tip for each test tube.
-Use a clean tube rack for next wash.
-Repeat wash **four times** in the following order: once with Wash Buffer, twice with ethanol, once with acetone.
-Carefully remove any residual acetone with a 100 µl pipette, using a fresh tip for each test tube.

8. Dry the silica pellets in open test tubes at 56°C for 10 ± 1 minutes in a heating block.

Note: Cover the test tubes with tissue to avoid contamination. Make sure that caps and corresponding test tubes can be identified, e.g., by marking caps and corresponding tubes.

9. -Check if test tubes are dry (i.e., the test tube must contain a white silica pellet which crumbles when the tube is flicked with the finger).

   -When dry, add 50 µl Elution Buffer to each test tube.
   -Vortex test tubes until the pellets are completely resuspended.

10. Leave the resuspended silica for 10 ± 1 minutes at 56°C to elute the nucleic acid.

    Note: Vortex the test tubes after five minutes to prevent silica from settling on the bottom.

11. Centrifuge test tubes 2 minutes at 10,000 g.

12. For each test tube:

    Transfer 5 µl of the supernatant nucleic acid to a fresh test tube. **Note:** If any resuspension of silica is suspected, spin the test tube again before transfer. Store the remaining supernatant with the silica pellet at 2-8°C for up to 14 days, or at –20°C, or preferably at –70°C, up to one year.

13. Transfer the test tubes containing the 5µl nucleic acid supernatant to the amplification lab area. Five microliters (5 µl) of supernatant nucleic acid must be used for the amplification procedure (continue with 6.3).

14. If the test tubes are not to be immediately used for amplification, store at –20°C, or preferably at –70°C, up to one year.

6.3. Nucleic Acid Amplification

Note: Steps 1 to 4 should be performed in a fume hood to reduce the risk of contamination; step 6 should preferably also be performed in a fume hood.

1. For each test tube (containing 5 µl supernatant nucleic acid): Add 10 µl of primer solution. Close the test tube.

2. Incubate test tubes for 5 ± 1 minutes at 65 ± 1°C.

3. Cool test tubes for 5 ± 1 minutes at 41 ± 0.5°C during which time proceed to step 4.

Amplification procedure

4. Mix prepared Enzyme Solution by gently flicking the closed tube.
   Note: **Do not vortex!**
   For each test tube:
   -Add 5 µl of enzyme solution.
   -Mix well by gently flicking the test tube with your finger.
   -Immediately return to 41 ± 0.5°C.
   -Incubate test tubes for a minimum of 5 minutes at 41 ± 0.5°C.
   Note: Avoid any unnecessary delay between this incubation and incubation in step 6 to prevent discontinuation of the amplification process.

5. Transfer test tubes to the detection lab area. Quick spin test tubes.

6. Incubate test tubes at 41 ± 0.5°C for 90 ± 5 minutes, using a water bath, heat block or equivalent.

7. Test tubes that are not to be immediately used for detection may be stored for up to 1 year at –20°C.

6.4. Nucleic Acid Detection

Note: Detection should preferably be performed in a fume hood to reduce the risk of contamination.

1. Prepare and label four fresh 1.5 ml tubes (1 = WT (wild type), 2 = Qa, 3 = Qb, 4 = Qc).
   -Vortex Bead-oligo (pink) until an opaque solution is formed.
   -Immediately after vortexing add 130µl of Bead-oligo to each tube (1-4). Recap the tubes.
   -Add 130µl of WT-ruthenium labeled probe (white) to tube 1. Recap the tube.
   -Add 130µl of Qa-ruthenium labeled probe (red) to tube 2. Recap the tube.
   -Add 130µl of Qb-ruthenium labeled probe (yellow) to tube 3. Recap the tube.
   -Add 130µl of Qc-ruthenium labeled probe (blue) to tube 4. Recap the tube.

2. For each amplified sample and control, prepare and label four fresh 5 ml polypropylene tubes (referred to as hybridization tubes, 1 = WT, 2 = Qa, 3 = Qb, 4 = Qc). Prepare one fresh 5 ml polypropylene tube for use as a blank (i.e., Detection Diluent instead of diluted amplified sample is added to this tube).

3. Vortex hybridization solutions until opaque. Using a repeating or standard pipette:
   -Add 20 µl of hybridization solution 1 to each hybridization tube 1 and to the blank.
   -Add 20 µl of hybridization solution 2 to each hybridization tube 2.
   -Add 20 µl of hybridization solution 3 to each hybridization tube 3.
   -Add 20 µl of hybridization solution 4 to each hybridization tube 4.

4. -For each amplified sample or control, add the indicated volume of Detection Diluent (see note below) to a fresh test tube, using a repeating or standard pipette.
   Note: The **volume** of Detection Diluent to be added **is specific to each lot**. The recommended volume is **indicated on the Detection box.**
   Refer to Section 7.2 “Troubleshooting” if dilution related problems are suspected.
   -Close the test tubes containing Detection Diluent.
   -Vortex and quick spin the amplified samples and controls. For each amplified sample or control:
   -Open one of the test tubes containing Detection Diluent.
   -Add 5 µl of amplified sample to the corresponding tube.
   -Close test tube.
   -Vortex and quick spin the test tubes.

   Note: Store remaining amplified sample material at –20°C for up to 12 months.

5. For each diluted amplified sample or control (diluted in step 4):
   -Add 5.0 µl diluted amplified sample or control to each of the four hybridization tubes, using a fresh pipette tip for each addition.
   Note: Avoid contact of the pipette tip with the inside wall of the hybridization tubes. The tip should only touch the hybridization solution in the tubes. Use waste containers with a cap for disposal of the pipette tips to reduce contamination risks.
   -Add 5.0 µl Detection Diluent to the blank.
   -Cover hybridization tubes and the blank with adhesive tape.
   -Mix hybridization tubes and the blank until an opaque solution is formed [either mix simultaneously, e.g. using a shaker (1,100 rpm), or vortex].

6. Incubate all hybridization tubes and the blank in a water bath for 30 ± 2 minutes at 41 ± 1°C for hybridization.
   Note: During hybridization, mix the hybridization tubes and blank every 10 minutes.

7. Add 300 µl Assay Buffer (refer to NucliSens® Reader Operator Manual) to each hybridization tube and the blank.

Note: The tubes can be identified by the color of their content (WT = colorless; Qa = light blue; Qb = medium blue; Qc = dark blue).

8. -Vortex the Instrument Reference Solution until opaque.
-Add 250 µl Instrument Reference Solution to a fresh 5 ml polypropylene tube.
-Place the tube with Instrument Reference Solution in position one (1) of the instrument carousel.
-Place the blank in position two (2) of the instrument carousel.
-Place the hybridization tubes (for each sample and control in the order WT, Qa, Qb, Qc) in the following positions of the instrument carousel (according to the sample positions in the worklist). For creating and editing the worklist, refer to NucliSens® Reader Operator Manual.

9. Run worklist (refer to NucliSens® Reader Operator Manual).
Note: A number of parameters specific to each lot (*v* parameter, input levels of Qa, Qb, Qc calibrator molecules, and checksum) are indicated on the Detection Module box. To run an assay, these parameters must be entered in the Sample Parameter window of the NucliSens® Reader software (refer to NucliSens® Reader Operator Manual).

6.5. Quality Control

The integrity of each individual result can be monitored by reference to the performance of the three internal calibrators. However, it is recommended that a high Positive Control, low Positive Control, and a Negative Control be included with the first run of each kit lot to verify product performance. Furthermore, the inclusion of a low Positive and a Negative Control with each subsequent run of the same kit lot is also recommended. Refer to the control manufacturer's package insert for instructions.

As with any new laboratory procedure, new operators should consider the use of additional controls to establish a high degree of confidence in performing the assay.

If using the NucliSens® HIV-1 RNA Controls, the expected range for the Positive Controls is stated in the package insert for the controls. The copy number per ml for each Positive Control should fall within the range indicated in the package insert. The Negative Control should give a less than lower detection limit result. If using commercially available controls or internally prepared controls, a typical acceptance range is approximately ± 3 standard deviations from the established copy level. If controls are not as expected, the run should be re-evaluated and a determination made as to whether the sample results are acceptable or should be repeated.

7. Results

7.1. Calculations

The NucliSens® Reader automatically calculates the number of WT HIV-1 RNA copies/ml in the plasma sample. Refer to the NucliSens® Reader Operator Manual for the viewing, printing, storing or retrieving of results.

The three calibrator signals (Qa, Qb and Qc) and total ECL signal (WT+Qa+Qb+Qc) are corrected for background noise and checked against a number of validity criteria (e.g. total signal, calibrator deviation from the calibration-curve). A sample is qualified as valid or invalid depending on the outcome of these validity checks. When the result has been qualified as valid, the calibrators are used to plot a calibration curve upon which the WT concentration is calculated. A result is given only if the sample's estimated number of copies falls within the range of 25 to approximately 5 million copies per ml.

The following **valid** results can be observed:

Result quantification	Example	Explanation
Below lower detection limit	<LDL	The number of WT copies is below the lower detection limit of the assay.
Within quantitative range	1000 copies/ml	Results are within the quantitative range.
Above upper limit of quantification	>>UDL copies/ml	The number of WT copies is more than 2 logs higher than the number of Qa input copies. Repeat the test using a reduced sample volume, so that the calculated number of copies will fall into the measurable range of the instrument.

7.2. Trouble shooting

7.2.1. Invalid results

Qc out of tolerance, Qc discarded
This message appears when the observed signal for calibrator Qc is out of range. For calculation of the wild type concentration, only calibrators Qa and Qb are used, but the result is still valid. An increased frequency of this message, however, may indicate a suboptimal performance of the assay. When this happens, check equipment (e.g., temperature of heating blocks or water baths) and procedures before starting a new run.

Redetect
If a sample is classified as invalid and the error messages **sensitivity shifted redetect, WT signal too high and Qa signal too high** appear, a volume of Detection Diluent other than the one indicated on the box of the Detection module should be used to achieve optimum results. This means that the dilution factor is to be adjusted (according to the formula below) in a way that the Qa signal will yield approximately 1,000,000 counts at estimated WT inputs of less than 10^4 copies. The detection should subsequently be repeated to make optimum use of the dynamic range of the NucliSens® Reader.

The new dilution factor = Current dilution factor x (Qb signal/200,000). (For current dilution factor refer to 6.4 “Nucleic Acid Detection,” step 4.)

The error messages below will appear in any of the three following cases:

1. Error message: **Sensitivity shifted redetect with other dilution factor**.

   Cause: WT <LDL and ECL signal for the calibrators too low.

   Calibration line is shifted, resulting in a sensitivity of the assay which is too low. Signals are lower than usual, possibly due to reduced efficiency of amplification or the selection of a dilution factor which is too high. To enhance the sensitivity, repeat detection using a lower dilution factor (calculated according to the formula above.)

2. Error message: **WT signal too high**.

   Cause: WT ECL signal too high and second excitation signal of WT not within the accepted range.

   WT signal is above the instrument’s upper detection limit. Repeat detection using a higher dilution factor (calculated according to the above formula), so that the WT signal will fall into the range of the instrument. If the WT signal is still above the instrument’s upper detection limit, then use less sample volume or dilute the sample with negative human plasma. Repeat the test starting from nucleic acid release.

3. Error message: **Qa signal too high**.

   Cause: Qa ECL signal too high and second excitation signal of Qa not within the accepted range. Qa signal is above the instrument's upper detection limit. Repeat detection using a higher dilution factor (calculated according to the above formula) so that the Qa signal will fall into the range of the instrument.

Retest
If the test for a sample is classified as invalid and the error messages mentioned below appear, the sample must be retested:

1. Error message: **Sensitivity shifted due to less efficient amplification**.

   Cause: WT < LDL and Qc < LDL.

   The sensitivity has shifted, possibly due to reduced efficiency of isolation or amplification. Repeat the test starting from nucleic acid release.

2. Error message: **Calculated copies for Qa and/or Qb out of tolerance**.

   Cause: Qa and/or Qb deviate from the calibration line.

   Other (non-instrument related) invalid situations appear when the assay fails (e.g., calibrator Qa and/or Qb out of range). In these cases, repeat the test starting from nucleic acid release.

3. Error message: **No amplification**.

   Cause: Total ECL signal too low.

   The assay failed, possibly due to reduced efficiency of isolation or amplification. Repeat the test starting from nucleic acid release.

Instrument error
A test for a sample is classified as **invalid** when the error message **instrument error** appears. All reasons for this error message are instrument-related (e.g. tube missing, power failure, no Assay Buffer etc.). Take appropriate corrective measures (refer also to the NucliSens® Reader Operator Manual) and repeat detection.

7.2.2. Use of secondary ECL signal

A secondary ECL signal is used for calculations when the primary signal for WT and/or Qa exceeds the instrument's upper detection limit. The validity of a linear relationship between primary and secondary ECL signals has been shown over a broad range. Therefore, redetection of an amplified sample is not required. If half the number of samples, or more, in a run have the remark "**Secondary ECL signal used**," use a new dilution factor as a standard for the next run(s) for an optimal use of the dynamic range of the ECL measurement. The new dilution factor is calculated according to the formula in 7.2.1.

7.3. Clinical implications of results

After HIV-1 infection and during the course of the disease, billions of virions are produced and destroyed daily.[17,18] After primary infection, the viral load reaches a "set point" in each individual. This set point appears to be between 10^2 and 10^6 HIV RNA copies/ml of plasma and remains relatively stable in asymptomatic patients over months and possibly years.[19]

The set point is strongly associated with rate of disease progression and time to death.[20-22] Subjects with baseline HIV RNA levels of <5,000 copies/ml have the lowest risk, whereas individuals with a virus level of about

50,000 copies/ml are at greatest risk of disease progression.[21] HIV RNA levels appear to be more predictive of progression than $CD4^+$ T-cell counts provided that the cell counts were higher than 350/µl.[21,23] Plasma HIV RNA levels can thus help to assess the onset of HIV-related symptoms which predict a high risk for opportunistic diseases to occur.

Viral load is also recommended as a guide, together with $CD4^+$ T-cell counts and the patient's physical condition, for the initiation of drug therapy.[24]

The value of HIV RNA levels in assessing response to therapy is also indicated. Several trials using combinations of anti-retroviral drugs have shown a strong reduction in the HIV RNA titer within several weeks.[25, 26] Protease inhibitor triple-drug combination results also in a prolonged reduction of the viral load.[27] In addition, these multi-drug combinations seem to reduce the clinical impact of viral drug resistance. Changing HIV RNA levels often paralleled the differences in clinical outcome and survival. However, more extensive studies and other drug regimens are required to substantiate this correlation.

8. Limitations

- Monitoring the effects of anti-retroviral therapy by serial measurement of plasma HIV-1 RNA has only been validated for patients with baseline viral loads = 93,000 copies/mL for pediatric patients and = 28,000 copies/mL for adult patients.
- The performance of the NucliSens® HIV-1 QT assay has only been adequately validated with HIV-1 subtype B specimens.
- NucliSens® HIV-1 QT assay is generally insensitive for detection of HIV-2 RNA. However, samples from individuals infected with HIV-2 may exhibit cross-reactivity in this assay.
- When testing specimens with viral load < 300 copies/mL, the user should consider the use of well-characterized reference materials titered from 150 copies/mL to 300 copies/mL, inclusive.

The NucliSens® HIV-1 QT 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 176 to $3.47X10^6$ copies/mL. The NucliSens HIV-1 QT assay can accurately detect a 0.3 log 10 (2-fold) or greater change in HIV-1 RNA for patients whose viral load is between 3500 copies/mL and 3,500,000 copies/mL. The NucliSens HIV-1 QT can accurately detect a 0.9 log 10 (9-fold) change in HIV-1 RNA for patients whose viral load is between 180 and 850 copies/ml.

Use of this product should be limited to personnel who have been trained in all aspects and techniques of the NucliSens® HIV-1 QT assay. Attention should be paid to adequate specimen collection, transporting, storage and processing procedures. Good laboratory techniques should be used in all procedures to ensure proper performance of this assay. Because of the high analytical sensitivity of this assay and the need to avoid all possible sources of contamination, extreme care should be taken to maintain the purity of the reagents and mixtures. All reagents should be monitored for purity. Discard any reagents that may be suspect or have exceeded their recommended shelf life. As with any diagnostic test, results from the NucliSens® HIV-1 QT assay should be interpreted with consideration of all clinical and laboratory findings.

A negative test result does not exclude the possibility of exposure to, or infection with, HIV-1.

The assay must be performed in strict accordance with the instructions in this package insert (especially those regarding contamination risks) and the NucliSens® Reader Operator Manual to obtain accurate, reproducible results.

Plasma should be used for determination of HIV-1 RNA levels. EDTA, citrate, or heparin may be used as anticoagulants.

In addition to quantitative HIV-1 RNA load, other virological or immunopathological factors may contribute to variable rates of $CD4^+$ T-cell counts and clinical outcome of the disease.[8]

9. Performance characteristics

9.1. Interfering substances

Elevated levels of lipids, bilirubin, and hemoglobin in specimens do not interfere with the quantitation of HIV-1 RNA by the NucliSens® HIV-1 QT assay. The presence of antinuclear antibodies in specimens has no effect on the performance of the assay. Specimens from individuals known to be positive for rheumatoid factor, from multiparous women and from pregnant women showed no detrimental effect on the quantitation of HIV-1 RNA. In addition, the presence of platelets in plasma does not appear to interfere with the performance of the NucliSens® HIV-1 QT assay.[30]

The following compounds have been found not to interfere with the quantitation of viral load by this assay: AZT, ddI, d4T, ddC, 3TC, indinavir, ritonavir, saquinavir, ganciclovir, acyclovir, zithromax-azithromycin, biaxin-clarithromycin, clofazamine, ethionamide, pentamidine, bactrin-trimethoprim sulfamethoxazole, dapsone, and diflucan.

Three anticoagulants, (EDTA, citrate, and heparin) have been tested with the assay. None have been found to exhibit any significant interference on the quantitation of HIV-1 RNA load by the NucliSens® HIV-1 QT assay.

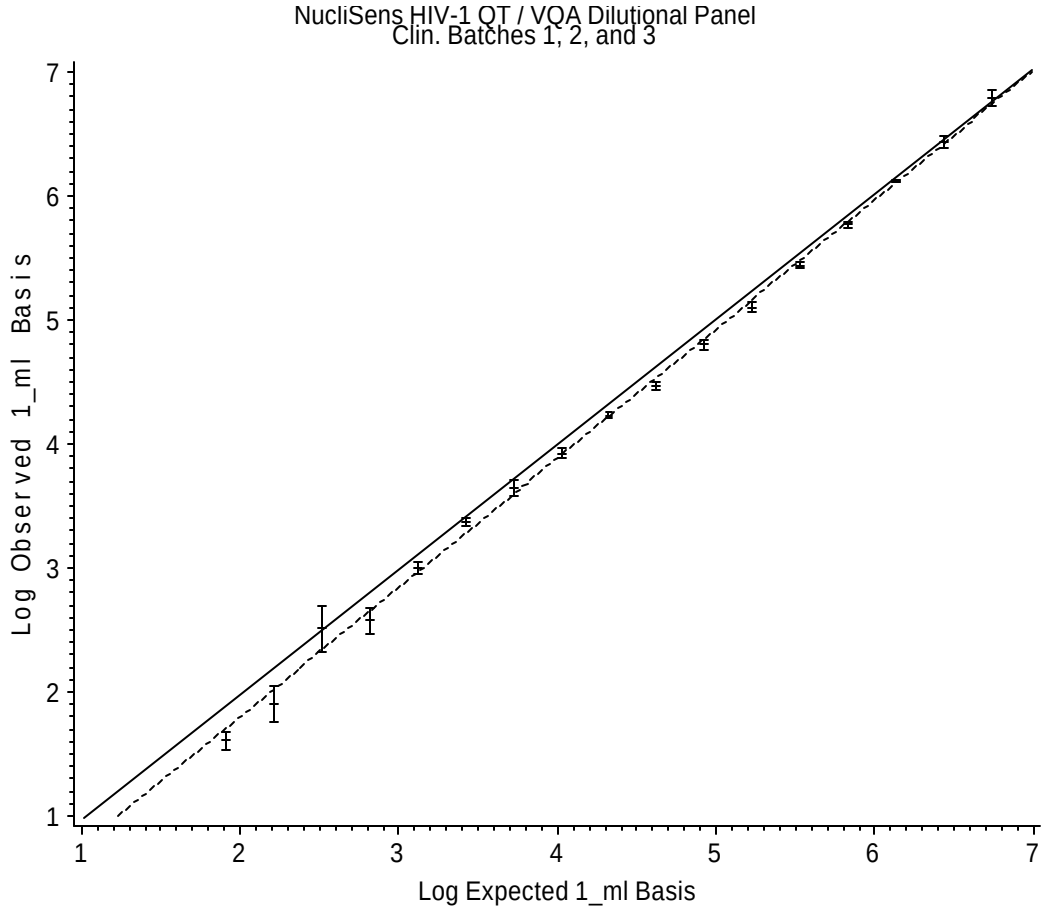
9.2. Linearity and Limit of Detection/Quantitation

The linear dynamic range (linearity) and limits of detection/quantitation of NucliSens® HIV-1 QT were assessed with specimens derived from a well-characterized HIV-1 RNA stock.

9.2.1. Linearity

Testing of diluted specimens derived from the HIV-1 RNA stock indicated a direct proportional relationship between the dilutions tested and the number of HIV-1 RNA copies reported. The linear range of the assay was determined using the combined data from three kit lots and linear regression analysis of the log observed HIV-1 RNA copies regressed on the log expected HIV-1 RNA copies. The performance of the assay was linear over a range of 51 to 5,390,000 HIV-1 RNA copies.

Figure 9-1: NucliSensÒ HIV-1 QT Linearity



y= -0.286+1.042x and R=0.993

9.2.2. Limits of Detection and Quantitation.

- The limit of detection (LOD) is defined as the lowest HIV-1 RNA input level where at least 95% of the tests produce a result indicative of reactivity of the input sample for HIV-1.
- The limit of quantitation (LOQ) is defined as the lowest HIV-1 RNA input level where at least 95% of the tests produce quantifiable (within linear range) results with reasonable accuracy and precision.

The limits of detection and quantitation for NucliSens® HIV-1 QT were determined by multiple testing at three sites of specimens containing HIV-1 RNA concentrations ranging from 659 to 41 copies/ml. Five specimens representing a range of HIV-1 RNA concentrations were selected and tested 72 times each using three lots of reagents.

Logistic regression was used to determine the relationship between the proportion detected and the log nominal input. The limit of detection at a 95% rate (LOD) was calculated to be 176 HIV-1 RNA copies/ml. This HIV-1 RNA input level for the Limit of Detection was verified by the results of the testing of panel members. For one panel member (nominal input = 164 HIV-1 RNA copies) the observed proportion of tests with positive results was 95.8%.

Table 9-1: Analysis of NucliSensÒ HIV-1 QT Detection Rates

Panel Member	HIV-1 RNA input	HIV-1 RNA log input	Total Tested	Number Tested Positive	Observed fraction positive	% CV
14	659	2.8189	72	72	1.0000	72
15	329	2.5172	72	72	1.0000	53
16	164	2.2148	72	69	0.9583	94
17	82	1.9138	72	51	0.7083	98
18	41	1.6128	72	38	0.5278	100

The 95% detection rate was determined by logistic regression analysis to be 176 copies/ml. The coefficients of variation (CV) for panel members 15 and 16 with input concentrations of 329 and 164 copies/ml, respectively, were 53% and 94% (Table 9-1). Based on these results the Limit of Quantitation has been determined to be equal to the Limit of Detection, i.e. 176 copies/ml.

Terminal Dilution of Clinical Specimens.

To evaluate the relative sensitivity of NucliSens® HIV-1 QT, dilutions of six clinical specimens from HIV-1 infected individuals were made with human plasma negative for HIV-1. In each case, the response in copy number was a function of the dilution factor, with a diminishing HIV-1 RNA copy number as dilution factor increased. The detection of HIV-1 RNA was similar for each of the three NucliSens® HIV-1 QT lots evaluated.

9.3. Comparison to FDA Approved Device (Roche AMPLICOR HIV-1 MONITOR ä)

Dilution panel members used for the linearity study, clinical specimens, and panels provided by CBER (FDA) and Boston Biomedica (BBI panel 709)) were tested with NucliSens® HIV-1 QT and the Roche AMPLICOR HIV-1 MONITOR™ Test, standard procedure. If available, samples with results reported as <400 copies/ml with the Standard MONITOR™ procedure were re-tested with the AMPLICOR UltraSensitive procedure. Results are shown in Table 9-2 below.

Table 9-2: Comparison of NucliSensÒ HIV-1 QT to Roche AMPLICOR HIV-1 MONITOR ä (Standard)

		Number of Specimens Reported with Copy Number	
Group	**Number**	**NucliSens® HIV-1 QT**	**Roche AMPLICOR HIV-1 MONITOR ä (Standard)**
VQA Dilutions	14	14	9
Clinical Specimens	76	76	63
BBI & CBER Panels	13	12	8
Totals (%)	103	102 (99%)	80 (78%)*

*Three of these not reported were above the Monitor™ upper limit of >750,000 copies/ml

Seventeen (17) specimens that were <400 in the Standard Roche AMPLICOR HIV-1 MONITOR™ Test were re-tested with the UltraSensitive Roche AMPLICOR HIV-1 MONITOR™ procedure. Of the 17 specimens, NucliSens® HIV-1 QT detected 16 (94%) and the UltraSensitive Roche AMPLICOR HIV-1 MONITOR™

procedure detected 15 (88%). One specimen was not detected by either test (CBER-B2). Results are presented in Table 9-3 below

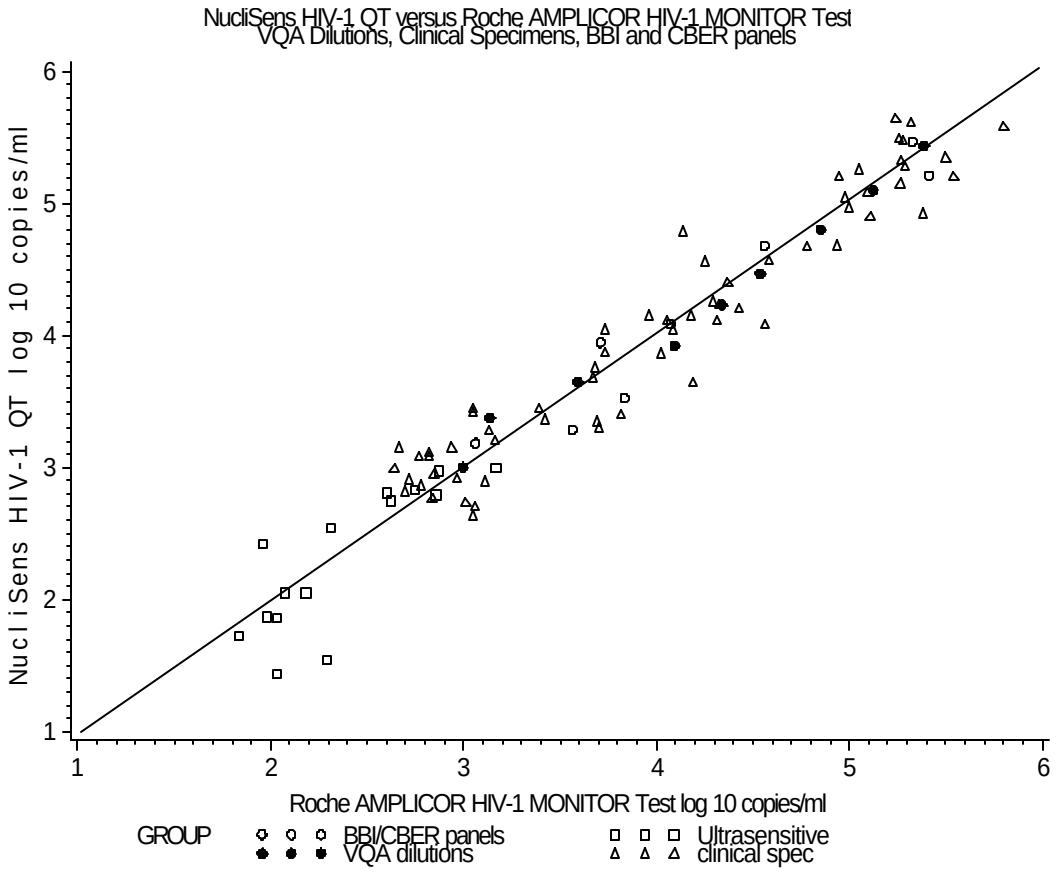
Table 9-3: Comparison of NucliSensÒ HIV-1 QT to Roche AMPLICOR HIV-1 MONITOR Test (Standard and UltraSensitive)

	NucliSensâ HIV-1 QT		Roche AMPLICOR HIV-1 MONITORÔ		
			Standard	UltraSensitive	
Specimen No.	Cp/ml	Log10	Cp/ml	Cp/ml	Log10
1	34	1.5315	<400	195	2.2900
2	920	2.9638	<400	744	2.8716
3	27	1.4314	<400	108	2.0334
4	72	1.8573	<400	95	1.9777
5	110	2.0414	<400	119	2.0755
6	260	2.4150	<400	92	1.9638
7	610	2.7853	<400	718	2.8561
8	670	2.8261	<400	563	2.7505
9	110	2.0414	<400	153	2.1847
10	970	2.9868	<400	1478	3.1697
11	630	2.7993	<400	402	2.6042
12	550	2.7404	<400	423	2.6263
13	71	1.8513	<400	108	2.0334
14	<LDL		<400	<50	
15	52	1.7160	<400	69	1.8388
16	160	2.2041	<400	<50	
17	340	2.5315	<400	207	2.3160

When NucliSens® HIV-1 QT and Roche AMPLICOR HIV-1 MONITOR Standard results were combined, an estimate of the Pearson product moment correlation, on a log œopy basis, was 0.96. The 95% confidence interval for the correlation is (0.945, 0.977). The results reported by both tests are highly correlated.

An X-Y plot was created to show the log base 10 HIV-1 RNA copy numbers for all data obtained with NucliSens® HIV-1 QT and Roche AMPLICOR HIV-1 MONITOR™ Test. The slope and intercept for this plot were 0.982 and 0.058, respectively. This further indicates the agreement of results obtained with both tests.

Figure 9-2: Log Results for the NucliSens® HIV-1 QT and Roche AMPLICOR HIV-1 MONITOR® Test are Plotted Compared to the Line of Perfect Agreement



9.4. Reproducibility

Reproducibility was determined by testing a panel (5 specimens) with varying levels of HIV-1 RNA with three lots at three sites by multiple technicians over four days. The variance components associated with run and replicate within run were used to estimate inter-assay variability and intra-assay variability, respectively. Inter-assay variability includes variability due to site, lot, and day. Intra-assay variability is replicate testing variability. The following table shows total inter-assay and intra-assay precision by specimen on an HIV-1 RNA copy basis.

Table 9-4: Per Specimen Precision Estimates - Copy Units

			TOTAL		INTER-ASSAY		INTRA-ASSAY	
SID	**N**	**MEAN**	**SD**	**CV**	**SD**	**CV**	**SD**	**CV**
SIII-1	72	3473611	1018421	29.3	832075	24.0	595469	17.1
SIII-2	72	356806	73806	20.7	52050	14.6	52691	14.8
SIII-3	72	47181	12729	27	10801	22.9	6857	14.5
SIII-4	72	3529	1257	35.6	998	28.3	774	21.9
SIII-5	71	373	383	102.8	234	62.8	305	81.7

As shown in Table 9-4, the NucliSens® HIV-1 QT was found to have relatively low variability. However, higher variability was observed for specimens with lower HIV-1 RNA concentrations.

9.5. Analytical Specificity

Analytical specificity was evaluated by testing a panel of specimens from individuals exhibiting the following infections or diseases, but serologically negative for HIV-1 infection. The majority of these specimens were tested with or without spiked HIV-1 RNA to assess false positive or false negative reactivity. Of the specimens tested, there was no evidence of false reactivity as shown in Table 9-5. The specificity was 100% across all sixteen specimen categories listed below with an overall 95% confidence interval of 97.9% - 100%. Overall recovery of spiked HIV-1 RNA ranged from 97% to 101%.

Table 9-5: Possible Interfering Specimens Tested

Specimen Category	Number of Specimens Tested	Unspiked Results[1]	Mean Spiked RNA Copies Recovered	Mean Recovery Relative to Expected[2]
HIV-2 positive	10	Not detected	24,500	97%
HTLV antibody positive	10	Not detected	33,500	100%
Herpes Simplex antibody positive	10	Not detected	28,100	100%
Icteric	10	Not detected	25,600	98%
Hemolyzed	10	Not detected	28,200	98%
Antinuclear Antibody	10	Not detected	24,900	99%
Lupus	10	Not detected	26,100	99%
Rheumatoid factor Positive	10	Not detected	35,000	100%
Specimens from Multiparous Woman	10	Not detected	26,500	98%
Specimens from Pregnant Woman	10	Not detected	24,900	97%
Syphilis (RPR) antibody positive	10	Not detected	51,500	Not Determined
Mycobacterium tuberculosis	10	Not detected	30,600	101%
Bacterially Contaminated	10	Not detected	69,300	Not Determined
Antibiotic/ Anti-Viral therapy	10	Not detected	60,410	Not Determined
Platelet	10	Not detected	26,400	101%
Lipemic	10	Not detected	30,600	99%
Summary Means	**160**	**Not detected**	**34,132**	**99%**

1 All specimens with no spiked HIV-1 virus were tested nonreactive (less than lower limit of detection).
2 Mean percent recovery was calculated by dividing the test result in log copy number with expected log copy number of the spiked specimens.

To evaluate the performance of NucliSens® HIV-1 QT assay on non-clade B HIV-1 subtypes, a panel of specimens with non-clade B subtypes obtained from BBI was tested using the NucliSens® HIV-1 QT assay and Roche AMPLICOR HIV-1 MONITOR Test (Standard). The results are presented in Table 9-6. Except for clade C, the NucliSens® HIV-1 QT assay showed improved detection efficiency as compared to the Roche AMPLICOR HIV-1 MONITOR Test. However, the difference of detection efficiency may not be statistically significant since only one specimen was tested for each subtype.

Table 9-6: NucliSensÒ HIV-1 QT and Roche AMPLICOR HIV-1 MONITORä Test Results with BBI Panel PRD201

	NucliSens® Results	Roche AMPLICOR HIV-1 MONITORä Results
Specimen	**Observed copy/ml (log)**	**Observed copy/ml (log)**
PRD201-01 Clade A	2,400 (3.3802)	Not detected
PRD201-02 Clade B	140,000 (5.1461)	149,360 (5.1742)
PRD201-03 Clade C	9,400 (3.9731)	23,861 (4.3776)
PRD201-04 Clade D	210,000 (5.3222)	394,638 (5.5961)
PRD201-05 Clade E	55,000 (4.7403)	12,387 (4.0929)
PRD201-06 Clade F	130,000 (5.1139)	50,423 (4.7026)
PRD201-07 Clade G	110,000 (5.0413)	8,401 (3.9243)
PRD201-08 Clade H	77,000 (4.8864)	1,508 (3.1784)
PRD201-09 (Diluent)	<LDL	Not Detected

<LDL = Less than Detectable Limit.

9.6. Clinical specificity

The clinical specificity of NucliSens® HIV-1 QT was assessed by testing a total of 502 plasma specimens collected from a low risk whole blood donor population. These specimens were non-reactive for antibodies to HIV-1 and HIV-2 as determined with an FDA licensed assay. The specificity of NucliSens® HIV-1 QT in this population was 100% (95% confidence limits of 99.27% to 100%).

9.7. Clinical Performance – Patient Prognosis and Drug Monitoring

9.7.1. Study Descriptions

The use of the assay was evaluated for patient prognosis and drug monitoring in conjunction with two clinical studies: 1) ACTG (AIDS Clinical Trial Group) Study 0152[31] and 2) the Delta Trial.[32]

ACTG 0152 evaluated the efficacy, safety, and long-term tolerance of zidovudine (AZT, also abbreviated as ZDV) or didanosine (ddI) as monotherapy or AZT and ddI in combination in a pediatric population. Children who were three months to eighteen years of age and who had received no or less than six weeks of previous anti-retroviral therapy and who had laboratory evidence of HIV infection were eligible for enrollment. Multiple specimens from 295 patients were tested with the assay. Two hundred eighty-seven (287) patients had reportable subsequent measurements for the calculation of proportion of progression to endpoint (Table 9-7). However, all 295 patients had reportable baseline HIV-1 RNA prior to randomization into intent-to-treat groups (Figures 9-3 and 9-4).

The Delta Trial was a multi-center study evaluating the efficacy and safety of combinations of the anti-retroviral drug AZT plus ddI or AZT plus zalcitabine (ddC) compared to AZT alone in HIV infected adults. The Delta Trial began in March, 1992 and continued through 1995. Participants in the Delta Trial included one hundred seventy-five (175) centers from Europe, Australia and New Zealand. All testing was performed at two sites on repository specimens from this study. In order to be eligible for the extended virology study, samples must have been stored frozen at –70°C or colder and must have been taken from individuals with a baseline sample and at least one later sample. Multiple samples from 1280 HIV infected participants in the Delta Trial were evaluated. Twelve hundred fifty-six (1256) had reportable subsequent measurement for the calculation of proportion of progression to endpoint (Table 9-8), of which eleven hundred thirty-three (1133) also had reportable baseline HIV-1 RNA prior to randomization into intent-to-treat groups (Figures 9-5 and 9-6).

9.7.2. Study Objectives

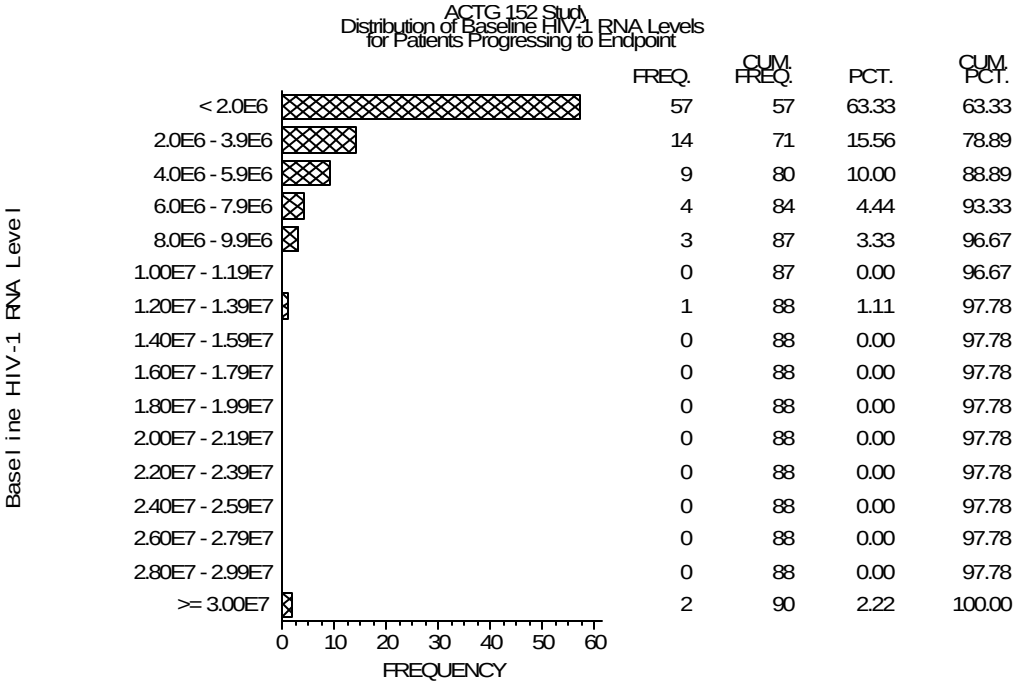
In each virology sub-study tied to these two investigations, the clinical utility was evaluated by 1) examining HIV-1 RNA measurement as a prognostic indicator of disease progression and 2) assessing the association, if any, of decreased HIV-1 RNA level with a change in patient therapy.

9.7.3. Patient Prognosis

To assess the association of RNA as measured by the assay with HIV-1 disease progression, a logistic function was used to model the probability of progressing to endpoint as a function of logarithm baseline HIV-1 RNA level. This model fit well, as measured by the Pearson Chi-Square Goodness-of-Fit Test for both the ACTG 152 study ($p=0.4304$) and Delta study ($p=0.7863$). For both studies, an overall association across the entire range of HIV-1 RNA concentrations was demonstrated with the test of slope coefficient ($p<0.001$). The estimate of slope was 0.976 for the ACTG 152 study and 0.999 for the Delta study. The estimates of slope for both studies are very similar and close to 1. Using the logistic model, the odds ratio was defined and used to calculate how much difference the assay can distinguish in terms of risk of clinical endpoints. For an individual with a viral load of 10,000 copies/ml, the risk of disease progression is 1.35 times as high as for an individual with a viral load of 5,000 copies/ml. These data suggest that a higher viral load is associated with an increased risk in both studies.

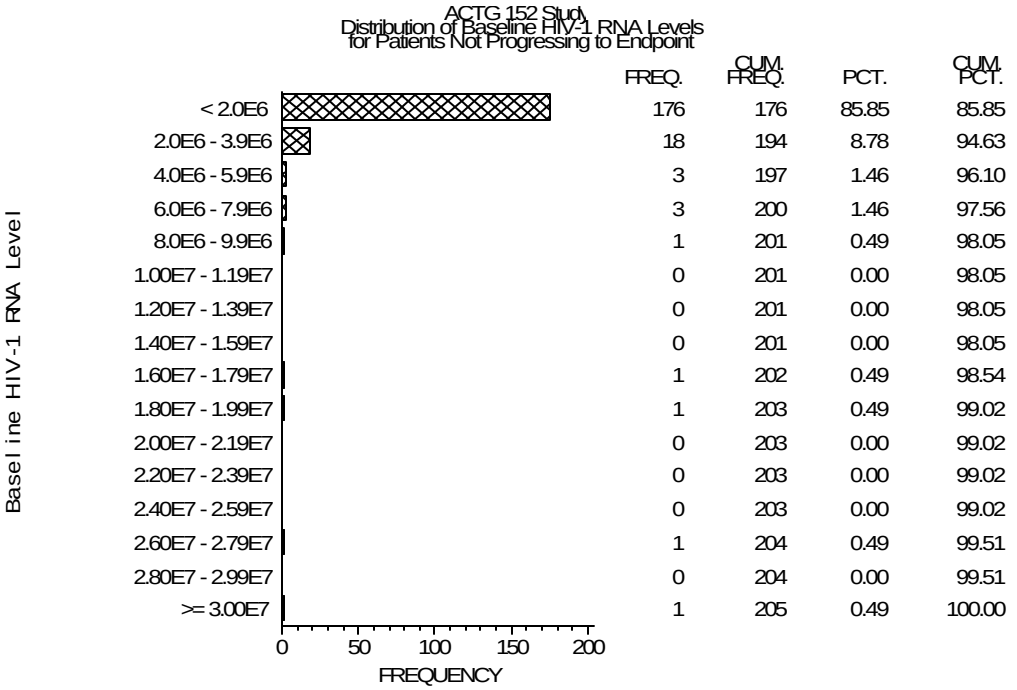
For each study, the distributions of actual baseline HIV-1 RNA levels for patients progressing and not progressing to endpoint were plotted. Figures 9-3 and 9-4 show the distribution of HIV-1 RNA levels for patients progressing and not progressing to endpoint, respectively, for the ACTG 152 study. Similarly, Figures 9-5 and 9-6 show the distribution of HIV-1 RNA levels for patients progressing and not progressing to endpoint, respectively, for the Delta Study.

Figure 9-3: Distribution of Baseline HIV-1 RNA Levels for Patients (N=90)Progressing to Endpoint – ACTG 152 Study



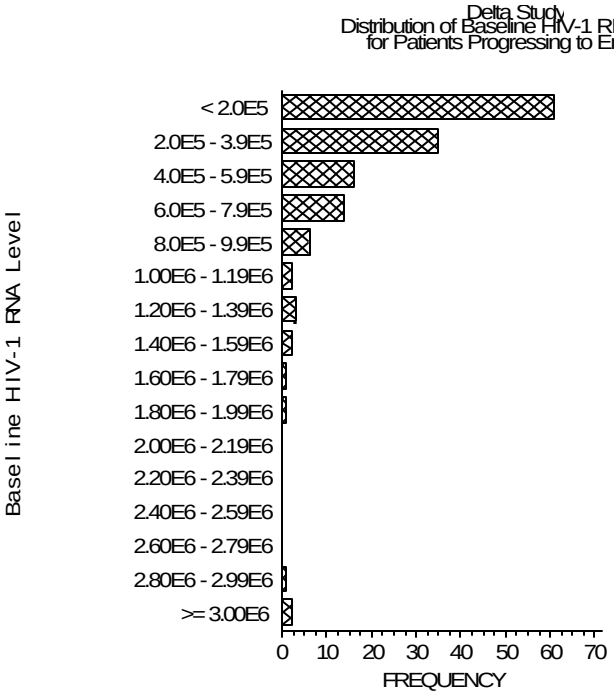
Baseline HIV-1 RNA Level	FREQ.	CUM. FREQ.	PCT.	CUM. PCT.
< 2.0E6	57	57	63.33	63.33
2.0E6 - 3.9E6	14	71	15.56	78.89
4.0E6 - 5.9E6	9	80	10.00	88.89
6.0E6 - 7.9E6	4	84	4.44	93.33
8.0E6 - 9.9E6	3	87	3.33	96.67
1.00E7 - 1.19E7	0	87	0.00	96.67
1.20E7 - 1.39E7	1	88	1.11	97.78
1.40E7 - 1.59E7	0	88	0.00	97.78
1.60E7 - 1.79E7	0	88	0.00	97.78
1.80E7 - 1.99E7	0	88	0.00	97.78
2.00E7 - 2.19E7	0	88	0.00	97.78
2.20E7 - 2.39E7	0	88	0.00	97.78
2.40E7 - 2.59E7	0	88	0.00	97.78
2.60E7 - 2.79E7	0	88	0.00	97.78
2.80E7 - 2.99E7	0	88	0.00	97.78
>= 3.00E7	2	90	2.22	100.00

Figure 9-4: Distribution of Baseline HIV-1 RNA Levels for Patients (N=205) Not Progressing to Endpoint – ACTG 152 Study



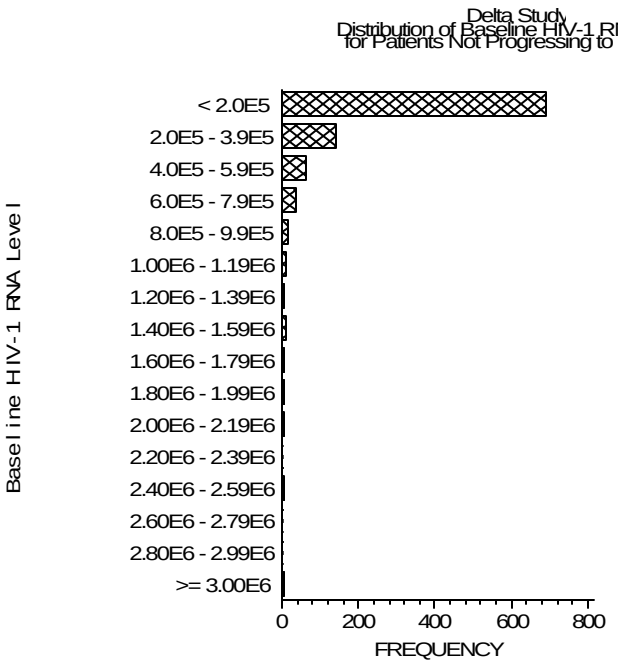
Baseline HIV-1 RNA Level	FREQ.	CUM. FREQ.	PCT.	CUM. PCT.
< 2.0E6	176	176	85.85	85.85
2.0E6 - 3.9E6	18	194	8.78	94.63
4.0E6 - 5.9E6	3	197	1.46	96.10
6.0E6 - 7.9E6	3	200	1.46	97.56
8.0E6 - 9.9E6	1	201	0.49	98.05
1.00E7 - 1.19E7	0	201	0.00	98.05
1.20E7 - 1.39E7	0	201	0.00	98.05
1.40E7 - 1.59E7	0	201	0.00	98.05
1.60E7 - 1.79E7	1	202	0.49	98.54
1.80E7 - 1.99E7	1	203	0.49	99.02
2.00E7 - 2.19E7	0	203	0.00	99.02
2.20E7 - 2.39E7	0	203	0.00	99.02
2.40E7 - 2.59E7	0	203	0.00	99.02
2.60E7 - 2.79E7	1	204	0.49	99.51
2.80E7 - 2.99E7	0	204	0.00	99.51
>= 3.00E7	1	205	0.49	100.00

Figure 9-5: Distribution of Baseline HIV-1 RNA Levels for Patients (N=144) Progressing to Endpoint – Delta Study



	FREQ.	CUM. FREQ.	PCT.	CUM. PCT.
< 2.0E5	61	61	42.36	42.36
2.0E5 - 3.9E5	35	96	24.31	66.67
4.0E5 - 5.9E5	16	112	11.11	77.78
6.0E5 - 7.9E5	14	126	9.72	87.50
8.0E5 - 9.9E5	6	132	4.17	91.67
1.00E6 - 1.19E6	2	134	1.39	93.06
1.20E6 - 1.39E6	3	137	2.08	95.14
1.40E6 - 1.59E6	2	139	1.39	96.53
1.60E6 - 1.79E6	1	140	0.69	97.22
1.80E6 - 1.99E6	1	141	0.69	97.92
2.00E6 - 2.19E6	0	141	0.00	97.92
2.20E6 - 2.39E6	0	141	0.00	97.92
2.40E6 - 2.59E6	0	141	0.00	97.92
2.60E6 - 2.79E6	0	141	0.00	97.92
2.80E6 - 2.99E6	1	142	0.69	98.61
>= 3.00E6	2	144	1.39	100.00

Figure 9-6: Distribution of Baseline HIV-1 RNA Levels for Patients (N=989) Not Progressing to Endpoint – Delta Study



	FREQ.	CUM. FREQ.	PCT.	CUM. PCT.
< 2.0E5	690	690	69.77	69.77
2.0E5 - 3.9E5	141	831	14.26	84.02
4.0E5 - 5.9E5	61	892	6.17	90.19
6.0E5 - 7.9E5	36	928	3.64	93.83
8.0E5 - 9.9E5	15	943	1.52	95.35
1.00E6 - 1.19E6	11	954	1.11	96.46
1.20E6 - 1.39E6	6	960	0.61	97.07
1.40E6 - 1.59E6	9	969	0.91	97.98
1.60E6 - 1.79E6	5	974	0.51	98.48
1.80E6 - 1.99E6	3	977	0.30	98.79
2.00E6 - 2.19E6	2	979	0.20	98.99
2.20E6 - 2.39E6	1	980	0.10	99.09
2.40E6 - 2.59E6	2	982	0.20	99.29
2.60E6 - 2.79E6	1	983	0.10	99.39
2.80E6 - 2.99E6	1	984	0.10	99.49
>= 3.00E6	5	989	0.51	100.00

The relationship between baseline RNA levels grouped by decile and the percent of subjects in each group progressing to endpoint in each decile was evaluated. Analyses were performed to look at differences in the probability of disease progression by sequentially testing combinations of data from different groups of deciles. With this approach, the proportion of subjects responding in decile 1 was contrasted with the proportion responding in the group formed by combining deciles 2-10. Next, the group formed by combining deciles 1 and 2 was contrasted against combined deciles 3-10, and so on. Tables 9-7 and 9-8 show decile, HIV-1 RNA copy number representation, and the numbers of individuals from the ACTG 152 and Delta Studies, respectively, progressing to endpoint and total number in each decile across all three treatment groups. The tables also show the contrast statement results of the test for differences between the proportions in sequential groupings of deciles. P-values associated with the contrasts for each study are not adjusted for multiple testing. These results show that the biggest increase in significance between successive decile groupings occurs between deciles two and three in both studies. Thus, in the ACTG 152 study, this jump occurs around 93,000 copies/mL; in the Delta around 28,000 copies/mL.

Table 9-7: Proportion to Endpoint by RNA Decile – ACTG 152 Study

Decile	Midpoint	Range	Progressing	N	Proportion	Cumulative Proportion	Sequential Testing	p value
1	<37000	<37000	3	29	0.10	0.10	1 vs 2-10	0.0183
2	65500	37000-93000	1	29	0.03	0.07	1-2 vs 3-10	0.0001
3	151500	93000-210000	7	31	0.23	0.12	1-3 vs 4-10	0.0001
4	275000	210000-340000	5	26	0.19	0.14	1-4 vs 5-10	0.0001
5	475000	340000-610000	8	29	0.28	0.17	1-5 vs 6-10	0.0001
6	855000	610000-1100000	11	33	0.33	0.20	1-6 vs 7-10	0.0001
7	1300000	1100000-1500000	12	25	0.48	0.23	1-7 vs 8-10	0.0001
8	1900000	1500000-2300000	11	28	0.39	0.25	1-8 vs 9-10	0.0001
9	3250000	2300000-4200000	14	29	0.48	0.28	1-9 vs 10	0.0001
10	>4200000	>4200000	18	28	0.64	0.31		
	Total		90	287	0.31			

Table 9-8: Proportion to Endpoint by RNA Decile – Delta Study

Decile	Midpoint	Range	Progressing	N	Proportion	Cumulative Proportion	Sequential Testing	p value
1	<12600	<12600	6	126	0.05	0.05	1 vs 2-10	0.0103
2	20300	12600-28000	6	126	0.05	0.05	1-2 vs 3-10	0.0001
3	38000	28000-48000	10	128	0.08	0.06	1-3 vs 4-10	0.0001
4	64000	48000-80000	10	134	0.07	0.06	1-4 vs 5-10	0.0001
5	96000	80000-112000	7	122	0.06	0.06	1-5 vs 6-10	0.0001
6	132000	112000-152000	15	118	0.13	0.07	1-6 vs 7-10	0.0001
7	186000	152000-220000	20	126	0.16	0.08	1-7 vs 8-10	0.0001
8	290000	220000-360000	22	125	0.18	0.10	1-8 vs 9-10	0.0001
9	480000	360000-600000	30	127	0.24	0.11	1-9 vs 10	0.0001
10	>600000	>600000	30	124	0.24	0.12		
	Total		156	1256	0.12			

Cox proportional hazards regression models, stratified by anti-retroviral treatment group, were used to estimate the capability of reductions in RNA to predict lowering the risk of disease progression at various reference weeks throughout the studies. Separate analyses were performed to include baseline HIV-1 RNA and later weeks' HIV-1 RNA and to include baseline $CD4^+$ count and later weeks' $CD4^+$ count as predictors. Only patients surviving without disease progression up to the reference week were included in each analysis. If no progression occurred within the time on study, the observation was treated as right censored. HIV-1 RNA copies and $CD4^+$ counts were log transformed. The hazard ratio in each model was defined as a 10-fold decrease in HIV-1 RNA or a 10-fold increase in $CD4^+$ count.

For each target week, a proportional hazards (PH) model was fit to the data. For the ACTG 152 study, a target week of 24 weeks was analyzed. Table 9-9 shows the results for the model including baseline HIV-1 RNA and change from baseline HIV-1 RNA to the target week HIV-1 RNA as predictors. A change in HIV-1 RNA from baseline to 24 weeks was significant (p=0.0042) in predicting a reduced risk of disease progression after the baseline HIV-1 RNA adjustment. For every 10-fold decrease in HIV-1 RNA, the hazard of disease progression decreases by 61%.

Table 9-9: ACTG 152 Study
Cox Proportional Hazards Model Results – Pediatric Population
Risk of Disease Progression vs log10(baseline RNA) and change from baseline RNA to 24 Weeks
Response: Time from Week to Endpoint for Patients Surviving Without Progression Through Given Week

Week	Model Covariate	Parameter Estimate	Standard Error	Wald Chi-Square	P-Value	Risk Ratio	Lower 95% Conf. Lim	Upper 95% Conf. Lim
24	-Log10(baseline RNA)	-0.9463	0.1845	26.3121	<0.0001	0.388	0.270	0.557
24	Change from baseline RNA to 24 wks	-0.6059	0.2118	8.1875	0.0042	0.546	0.360	0.826

Table 9-10 shows the results for the model including baseline $CD4^+$ count and change from baseline $CD4^+$ count to the target week $CD4^+$ count as predictors. A change in $CD4^+$ count from baseline to 24 weeks was significant (p=0.0001) in predicting a reduced risk of disease progression after the baseline $CD4^+$ count adjustment. For every 10-fold increase in $CD4^+$ count, the hazard of disease progression decreases by 84%.

Table 9-10: ACTG 152 Study
Cox Proportional Hazards Model Results – Stratified by Treatment Therapy – Pediatric Population
Risk of Disease Progression vs log10(baseline $CD4^+$) and change from baseline $CD4^+$ to 24 Weeks
Response: Time from Week to Endpoint for Patients Surviving Without Progression Through Given Week

Week	Model Covariate	Parameter Estimate	Standard Error	Wald Chi-Square	P-Value	Risk Ratio	Lower 95% Conf. Lim	Upper 95% Conf. Lim
24	Log10(baseline CD4)	-1.8300	0.2925	39.1409	<0.0001	0.160	0.090	0.285
24	Change from baseline CD4 to 24 wks	-1.5180	0.3930	14.9230	0.0001	0.219	0.101	0.473

For the Delta study, target weeks of 8 and 16 weeks were analyzed. Table 9-11 shows the results for the model including baseline HIV-1 RNA and change from baseline HIV-1 RNA to the target week HIV-1 RNA as

predictors. A change in HIV-1 RNA from baseline to 8 or 16 weeks was significant (p<0.0001) in predicting a reduced risk of disease progression after the baseline HIV-1 RNA adjustment. For every 10-fold decrease in baseline HIV-1 RNA, the hazard of disease progression decreases by 75%.

Table 9-11: Delta Study
Cox Proportional Hazards Model Results – Stratified by Treatment Therapy – Adult Population
Risk of Disease Progression vs log10(baseline RNA) and change from baseline RNA to Given Weeks
Response: Time from Week to Endpoint for Patients Surviving Without Progression Through Given Week

Week	Model Covariate	Parameter Estimate	Standard Error	Wald Chi-Square	P-Value	Risk Ratio	Lower 95% Conf. Lim	Upper 95% Conf. Lim
8	-Log10(baseline RNA)	-1.4004	0.2019	48.1301	<0.0001	0.247	0.166	0.366
8	Change from baseline RNA to 8 wks	-1.5727	0.1894	68.9804	<0.0001	0.207	0.143	0.301

Week	Model Covariate	Parameter Estimate	Standard Error	Wald Chi-Square	P-Value	Risk Ratio	Lower 95% Conf. Lim	Upper 95% Conf. Lim
16	-Log10(baseline RNA)	-1.3996	0.2462	32.3143	<0.0001	0.247	0.152	0.400
16	Change from baseline RNA to 16 wks	-1.2657	0.2116	35.7955	<0.0001	0.282	0.186	0.427

Table 9-12 shows the results for the model including baseline $CD4^+$ count and change from baseline $CD4^+$ count to the target week $CD4^+$ count as predictors. A change in $CD4^+$ count from baseline to 8 or 16 weeks was significant (p=0.0304 and p=0.0185, respectively) in predicting a reduced risk of disease progression after the baseline $CD4^+$ count adjustment. For every 10-fold increase in $CD4^+$ count, the hazard of disease progression decreases by 96-97%.

Table 9-12: Delta Study
Cox Proportional Hazards Model Results – Stratified by Treatment Therapy – Adult Population
Risk of Disease Progression vs log10(baseline CD4$^+$) and change from baseline CD4$^+$ to Given Weeks
Response: Time from Week to Endpoint for Patients Surviving Without Progression Through Given Week

Week	Model Covariate	Parameter Estimate	Standard Error	Wald Chi-Square	P-Value	Risk Ratio	Lower 95% Conf. Lim	Upper 95% Conf. Lim
8	Log10(baseline CD4)	-3.2414	0.3054	112.6384	<0.0001	0.039	0.021	0.071
8	Change from baseline CD4 to 8 wks	-1.0266	0.4741	4.6882	0.0304	0.358	0.141	0.907

Week	Model Covariate	Parameter Estimate	Standard Error	Wald Chi-Square	P-Value	Risk Ratio	Lower 95% Conf. Lim	Upper 95% Conf. Lim
16	Log10(baseline CD4)	-3.7019	0.3852	92.3740	<0.0001	0.025	0.012	0.052
16	Change from baseline CD4 to 16 wks	-1.1009	0.4673	5.5507	0.0185	0.333	0.133	0.831

For each target week, another proportional hazards (PH) model was fit to the data. This model included baseline HIV-1 RNA, change from baseline HIV-1 RNA to the target week HIV-1 RNA, baseline CD4$^+$ count, and change from baseline CD4$^+$ count to the target week CD4$^+$ count as predictors. For the ACTG 152 study, a target week of 24 weeks was analyzed. Table 9-13 shows the results for the model including baseline HIV-1 RNA, change from baseline HIV-1 RNA to the target week HIV-1 RNA, baseline CD4$^+$ count, and change from baseline CD4$^+$ count to the target week CD4$^+$ count as predictors. A change in HIV-1 RNA from baseline to 24 weeks was marginally significant (p=0.0703) in predicting a reduced risk of disease progression after the baseline HIV-1 RNA adjustment and adjustment for CD4$^+$ count and 24 week change in CD4$^+$ count. For every 10-fold decrease in baseline HIV-1 RNA, the hazard of disease progression decreases by 52%.

Table 9-13: ACTG 152 Study
Cox Proportional Hazards Model Results – Stratified by Treatment Therapy – Pediatric Population
Risk of Disease Progression vs log10(baseline RNA), change from baseline RNA to 24 Weeks, log10(baseline CD4$^+$), and change from baseline CD4$^+$ to 24 Weeks
Response: Time from Week to Endpoint for Patients Surviving Without Progression Through 24 Weeks

Week	Model Covariate	Parameter Estimate	Standard Error	Wald Chi-Square	P-Value	Risk Ratio	Lower 95% Conf. Lim	Upper 95% Conf. Lim
24	-Log10(baseline RNA)	-0.7332	0.1934	14.3665	0.0002	0.480	0.329	0.702
24	Change from baseline RNA to 24 wks	-0.4092	0.2261	3.2760	0.0703	0.664	0.426	1.034
24	Log10(baseline CD4)	-1.4861	0.2991	24.6853	<0.0001	0.226	0.126	0.407
24	Change from baseline CD4 to 24 wks	-1.3443	0.3954	11.5596	0.0007	0.261	0.120	0.566

For the Delta study, target weeks of 8 and 16 weeks were analyzed. Tables 9-14 and 9-15 show the results for the model including baseline HIV-1 RNA, change from baseline HIV-1 RNA to the target week HIV-1 RNA, baseline $CD4^+$ count, and change from baseline $CD4^+$ count to the target week $CD4^+$ count as predictors. A change in HIV-1 RNA from baseline to 8 or 16 weeks was significant (p=0.0001) in predicting a reduced risk of disease progression after the baseline HIV-1 RNA adjustment and adjustment for $CD4^+$ count and 8 or 16 week change in $CD4^+$ count. For every 10-fold decrease in baseline HIV-1 RNA, the hazard of disease progression decreases by 64-69%.

Table 9-14: Delta Study
Cox Proportional Hazards Model Results – Stratified by Treatment Therapy – Adult Population
Risk of Disease Progression vs log10 (baseline RNA), change from baseline RNA to 8 Weeks, log10(baseline $CD4^+$), and change from baseline $CD4^+$ to 8 Weeks
Response: Time from Week to Endpoint for Patients Surviving Without Progression Through 8 Weeks

Week	Model Covariate	Parameter Estimate	Standard Error	Wald Chi-Square	P-Value	Risk Ratio	Lower 95% Conf. Lim	Upper 95% Conf. Lim
8	-Log10 (baseline RNA)	-1.1808	0.2134	30.6045	0.0001	0.307	0.202	0.467
8	Change from baseline RNA to 8 wks	-1.4035	0.2038	47.4392	0.0001	0.246	0.165	0.366
8	Log10 baseline CD4)	-2.8880	0.3509	67.7321	0.0001	0.056	0.028	0.111
8	Change from baseline CD4 to 8 wks	-0.0940	0.5236	0.0322	0.8575	0.910	0.326	2.540

Table 9-15: Delta Study
Cox Proportional Hazards Model Results – Adult Population
Risk of Disease Progression vs log10 (baseline RNA), change from baseline RNA to 16 Weeks, log10(baseline $CD4^+$), and change from baseline $CD4^+$ to 16 Weeks
Response: Time from Week to Endpoint for Patients Surviving Without Progression Through 16 Weeks

Week	Model Covariate	Parameter Estimate	Standard Error	Wald Chi-Square	P-Value	Risk Ratio	Lower 95% Conf. Lim	Upper 95% Conf. Lim
16	-Log10 (baseline RNA)	-1.0224	0.2589	15.5925	0.0001	0.360	0.217	0.598
16	Change from baseline RNA to 16 wks	-0.8819	0.2242	15.4682	0.0001	0.414	0.267	0.642
16	Log10 (baseline CD4)	-3.1842	0.4185	57.9052	0.0001	0.041	0.018	0.094
16	Change from baseline CD4 to 16 wks	-0.7114	0.5046	1.9872	0.1586	0.491	0.183	1.320

9.7.4. Measuring Response to Anti-retroviral Therapy

The association between HIV-1 RNA levels and survival time during the study without disease progression was assessed using Kaplan-Meier product limit survival curves. For this analysis the population was nominally divided into lower, middle, and upper thirds based on RNA levels. In the pediatric population, percent survival without progression referenced from week 24 was plotted as function of time in study based on the most recent RNA level prior to week 24. Only subjects who had not experienced disease progression prior to week 24 were included in the analysis. A similar analysis was performed with the adult population with a reference point of 8 weeks. A separate set of curves was generated for each treatment group.

Figures 9-7, 9-8, and 9-9 show the Kaplan-Meier curves for survival without progression to endpoint for the 3 anti-retroviral treatments studied with the pediatric population. Figures 9-10, 9-11, and 9-12 show similar curves for the 3 anti-retroviral treatments studied with the adult population. These figures demonstrate that, in each treatment group, subjects in the lowest RNA third survived longest without progression while subjects in the highest third progressed to endpoint most rapidly.

In another analysis, percent median change from baseline RNA by drug treatment was plotted versus weeks on study. Figure 9-13 plots percent change for the pediatric population study; Figure 9-14 shows a similar plot for the adult population study. In each study, the greatest and most enduring changes are seen in the treatment arms reflecting combination therapies.

Figure 9-7: ACTG 152
Kaplan-Meier Estimated Survival by Third of RNA Level at Week 24

Patients Surviving without Disease Progression Though Week 24
Pediatric Population Study - Treatment Therapy: ZDV

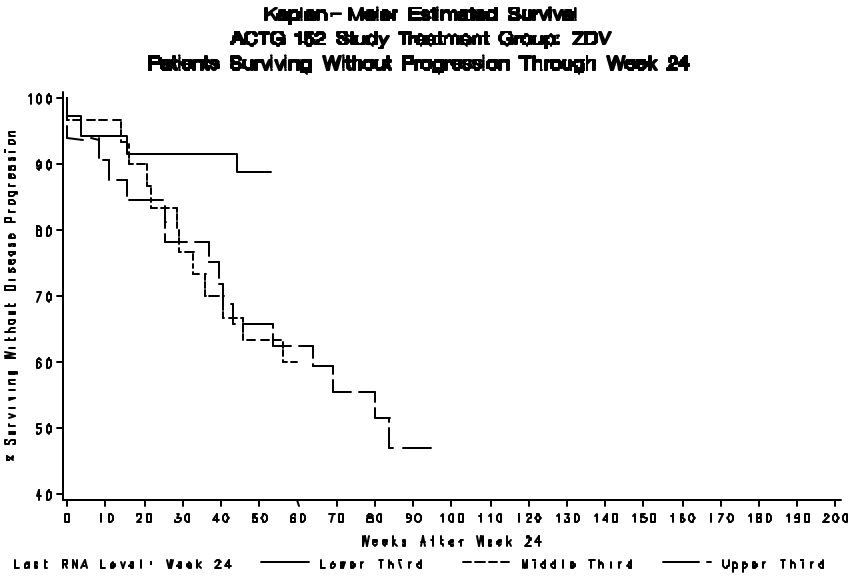


Figure 9-8: ACTG 152 Kaplan-Meier Estimated Survival by Third of RNA Level at Week 24

Patients Surviving without Disease Progression Though Week 24
Pediatric Population Study - Treatment Therapy: ddI

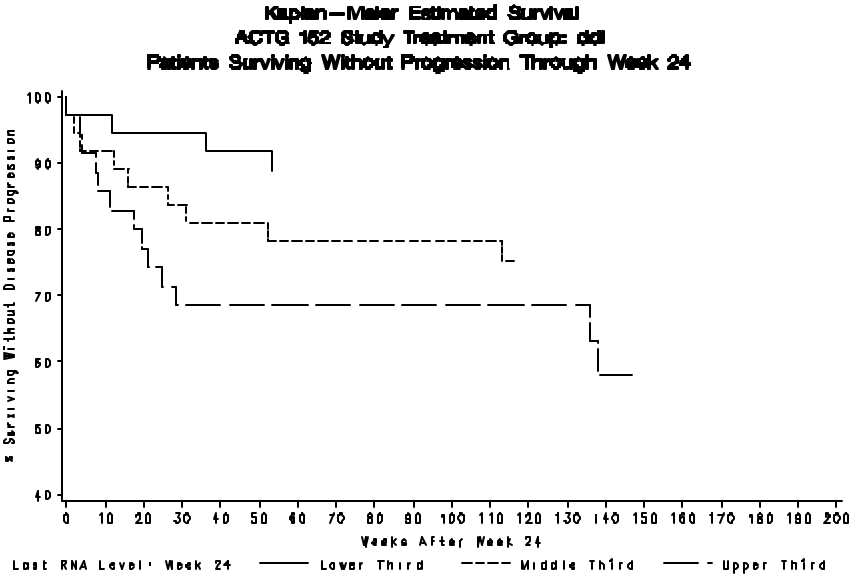


Figure 9-9: ACTG 152 Kaplan-Meier Estimated Survival by Third of RNA Level at Week 24

Patients Surviving without Disease Progression Though Week 24
Pediatric Population Study - Treatment Therapy: ZDV+ ddI

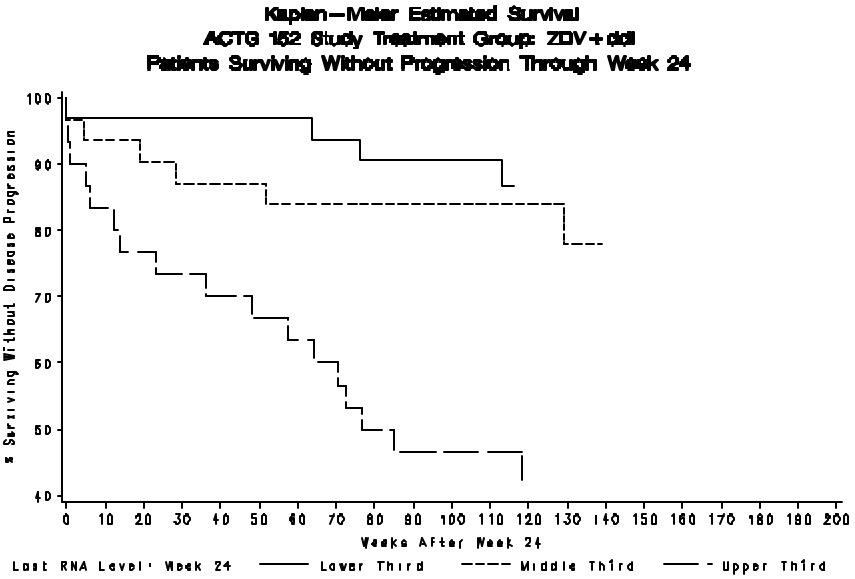


Figure 9-10: Delta Trial Kaplan-Meier Estimated Survival by Third of RNA Level at Week 8

Patients Surviving without Disease Progression Though Week 8
Adult Population Study - Treatment Therapy: AZT

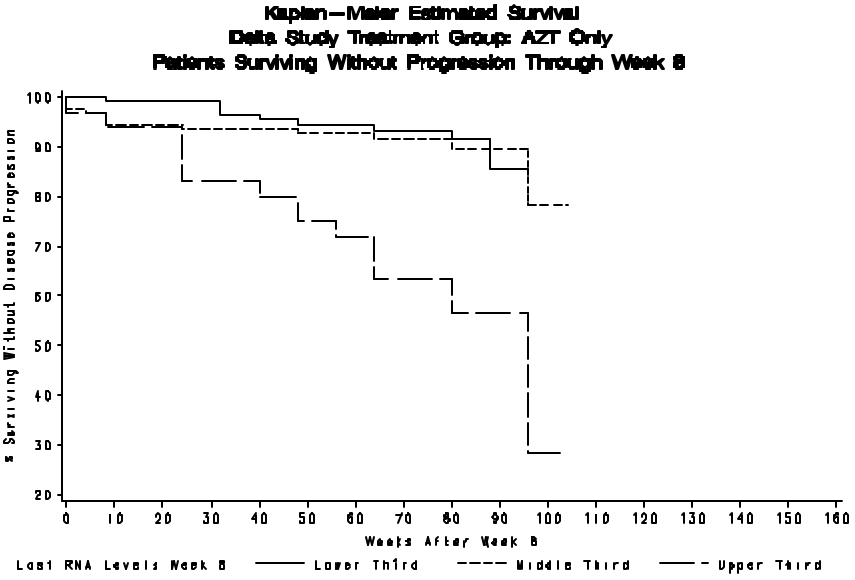


Figure 9-11: Delta Trial Kaplan-Meier Estimated Survival by Third of RNA Level at Week 8

Patients Surviving without Disease Progression Though Week 8
Adult Population Study - Treatment Therapy: AZT+ddI

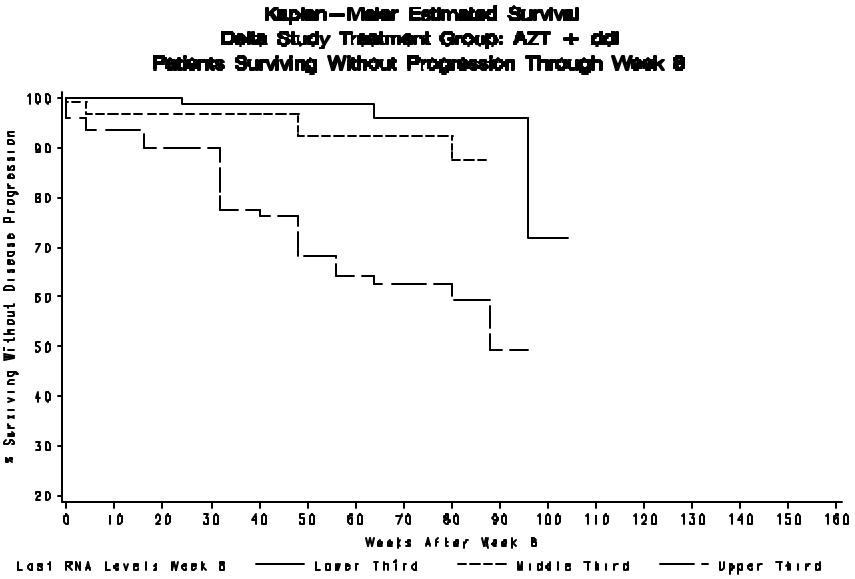


Figure 9-12: Delta Trial Kaplan-Meier Estimated Survival by Third of RNA Level at Week 8

Patients Surviving without Disease Progression Though Week 8
Adult Population Study - Treatment Therapy: AZT+ddC

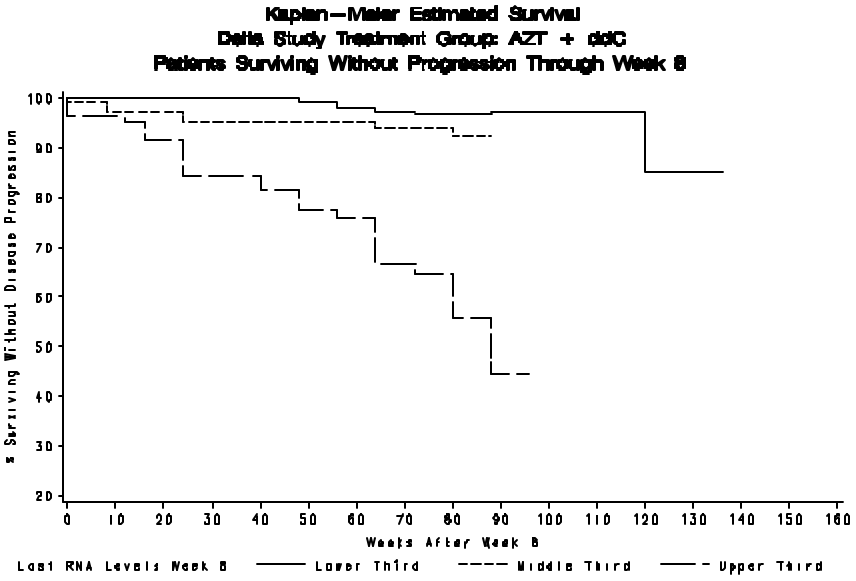


Figure 9-13: ACTG 152
Percent Change in Median RNA Level from Baseline RNA Level as A Function of Weeks on Study By Treatment Therapy

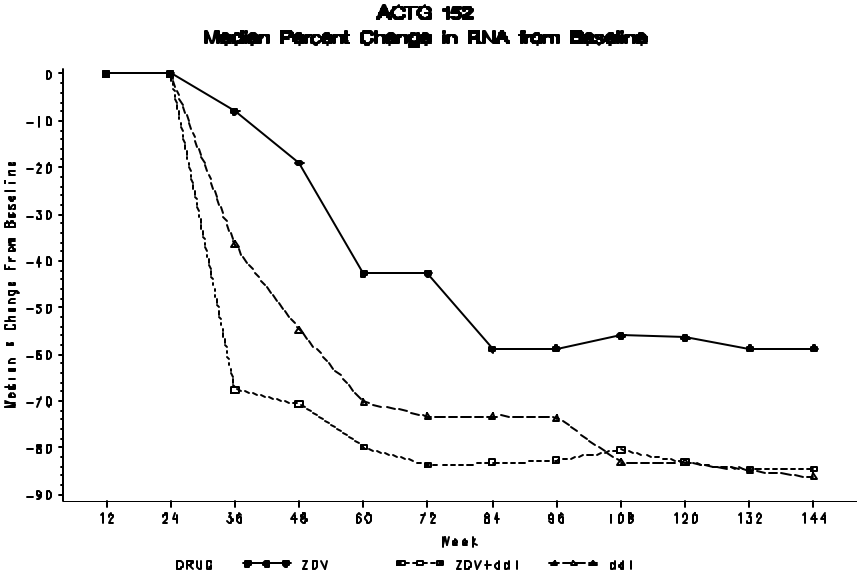
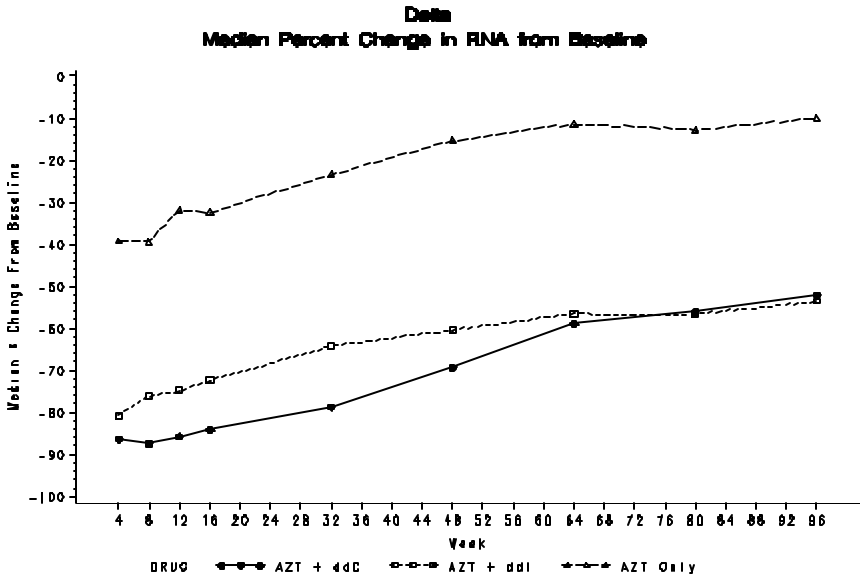


Figure 9-14: Delta Trial
Percent Change in Median RNA Level from Baseline RNA Level as A Function of Weeks on Study
By Treatment Therapy



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