NUCLEIC ACID AMPLIFICATION TECHNIQUES AND EVALUATION OF RNA QUANTITATION ASSAYS IN HIV 1 SUBTYPE B VIRUS

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A number of molecular techniques using either target amplification reactions (ie., Lcx, NASBA, PCR, TMA) or signal amplification reactions (ie., bDNA, Hybrid Capture) have been recently developed to quantify circulating HIV RNA virions in plasma of infected persons. The levels of patient's HIV 1 RNA (HIV viral load) are valuable prognostic markers of disease progression and an important tool for the management of infected individuals (1).

Currently there are three commercially available quantitative HIV RNA tests, Branched DNA (bDNA, Quantiplex HIV Assay), NucliSens (NASBA HIV 1 QT Assay), and the only FDA-approved RT-PCR (Amplicor HIV 1 Monitor Test) being used in routine patient management. These assays differ in their requirements for sample volume, specimen preparation, methods of amplification and detection (2). There are strengths and weaknesses to each of the assays, therefore there is probably no one assay that's best suited to all situations.

AMPLICOR HIV 1 Monitor (Roche Diagnostic Corporation, Indinapolis, IN)

This test is based on reverse-transcriptase mediated nucleic acid amplification using polymerase chain reaction technique. HIV 1 RNA is first reverse transcribed to a double-stranded DNA, and using it as a template together with HIV gag directed primer pairs, it is amplified in a PCR reaction (3). Large numbers of DNA copies are generated by PCR. Detection of these amplified products is done by hybridization of the amplified products to oligonucleotide probes specific to HIV RNA and the internal control using an EIA format. A unique feature of this assay is that it permits reverse transcription and amplification of HIV 1 and an internal standard (QS RNA) to occur simultaneously. Quantitation standard is incorporated into each reaction so differences in amplification efficiency caused by sample inhibitors and variability in reaction conditions or thermal cycling can be normalized. AmprErase (UNG, uracil-N-glycolylase) and dUTP are also incorporated into the reactions to ensure that any carryover of DNA from previous amplifications will not compromise quantitation.

HIV 1 RNA levels in the test specimen are determined by comparing to the absorbence obtained for the quantitation standard. Therefore, the QS compensates for any effects of inhibition and controls for the amplification process to allow the accurate quantitation of each specimen. The HIV copy number is determined by extrapolating the adjusted HIV signal from the internal control standard curve. The quantitative assay has a three log dynamic range for detection. Total elapsed time of the assay is less than eight hours. RT-PCR tests require separate work areas for specimen preparation, amplification and detection.

Quantiplex HIV 1 RNA Assay (Branched DNA, bDNA) (Chiron Corporation, Emeryville, CA)

Quantiplex HIV 1 RNA Assay, a branched DNA technology, is a solution-phase sandwich nucleic acid hybridization assay employing bDNA molecules (4). The specimen is treated with lysing reagent and the liberated viral nucleic acid is then hybridized in solution using two sets of oligonucleotide target probes. One set of oligonucleotide probes serves as the capture probe (located on the surface of microwell plate) which hybridizes specifically with HIV RNA and causes the viral RNA to be bound to the microplate. The second set of oligonucleotide probes serves as the target probe that binds the HIV RNA as well as hybridizing specifically with portions of the branched DNA molecules which attribute to the increased level of signal amplification. The bDNA molecules act as amplifiers by binding with alkaline phosphatase labeled probes. In this manner, the signal generated from the probe-target complex is greatly amplified for detection and quantification of viral RNA. Finally, the addition of a chemilumonescent substrate and the resulting signal is read by a luminometer. The relative light unit (RLU) generated is proportionally correlated with amount of HIV RNA in the sample. HIV 1 RNA copies are calculated using a standard curve generated from a set of four external HIV control standards. Branched DNA is an overnight assay. The bDNA method is less labor intensive than some of the other assays, and it appears to be easier to integrate into the laboratory that has no previous experience with molecular methods.

NucliSens HIV 1 RNA QT (Nucleic Acid Sequence Based Amplification, NASBA)

The NASBA assay is also a nucleic acid amplification method, but it differs from PCR. NASBA uses an isothermal target amplification with the coordination of simultaneous reaction of three enzymes, Avian myeloblastosis virus reverse transcriptase, Rnase H, and T7 RNA polymerase. The steps of the NASBA process take place in one reaction tube at a single temperature (41oC). A unique feature of the test is that one of the oligonucleotide primers contains a T7 RNA polymerase promotor which binds with HIV 1 RNA. During the reaction, RT generates a single DNA copy of the target HIV RNA. The Rnase H cuts off the RNA portion of the DNA:RNA hybrid, and the second ologinucleotide primer anneals to the remaining DNA strand. The DNA dependent DNA polymerase activity of RT can then extend from the second primer and produce a double stranded DNA copy of the original target RNA with an intact T7 RNA polymerase promotor. This T7 promotor is recognized by the T7 RNA polymerase which initiates transcription of larger amounts of single-stranded HIV RNA (5).

Internal control for the NASBA assay requires addition of three synthetic RNAs (Qa, Qb, Qc) of known high, medium and low concentration to the lysis buffer together with the target nucleic acid. Detection of HIV 1 RNA in a sample is based on the NASBA QR System electrochemiluminescence principle. For quantification and detection of the amplified products, aliquots of the amplified sample are added to four hybridization solutions, each specific for one of the amplicates. The respective amplicates are then hybridized with bead-oligo and ruthenium-labeled probe. The light emitted by the hybridized ruthenium-labeled probe is proportional to the amount of amplicate. Calculation based on the relative amounts of the four amplicates reveals the original amount of HIV 1 from the sample. Similar to the Amplicor Test, reagent preperation, specimen preparation, amplification and detection should be performed in separate areas of the laboratory.

Since the recent introduction of highly active combination therapy for treating HIV infected individuals, the HIV 1 RNA levels in plasma from many patients frequently dropped below

the quantitation limit (<400-500 copies/mL) of currently available tests. To achieve increased sensitivity, companies have come up with several modifications or improvements during the past year.

For the Amplicor HIV 1 Monitor Test, a modified specimen preparation procedure that allows input of RNA from 10-fold more plasma per amplification reaction was developed. This ultrasensitive method allows the accurate quantitation of plasma HIV 1 RNA levels as low as 50 copies/mL. The linear range of this test is 50 - 50,000 copies/mL of plasma (6). For Quantiplex HIV RNA version 3.0, the amplification molecules have been redesigned by incorporation of the non-natural bases isocytidine and isoguanosine to reduce their hybridization potential to all non-target nucleic acids. As a result, the sensitivity of the test was greatly improved and the linear range of the test is 50 - 500,000 copies/mL (7). NASBA HIV 1 QT assay has a very unique specimen preparation step that can accommodate a wide range of anticoagulants as well as a variety of specimen types including plasma, whole blood, CSF, seminal fluid, cervicovaginal lavage, and tissue. The nucleic acid isolation is based on the binding of nucleic acid to silica oxide particles. These silica particles carry the bound viral RNA through a series of washing steps. The HIV RNA is eluted in a small volume of buffer. With this feature, NASBA can accomodate specimen volumes of 10uL to 2.0mL. The dynamic range of this test is between 30 - 10 x 107 copies/mL.

The Virology Committee of the AIDS Clinical Trials Groups (ACTG) in the Division of AIDS, National Institute of Allergy and Infectious Diseases has developed a Quality Assurance Program (QAP) to assess the laboratory and kit performance of HIV 1 RNA measurements suitable for clinical studies. The primary goal of our study was to ensure laboratory quality and provide a set of HIV 1 RNA standards and controls which could be used for all assays. The main objective of QAP was to ascertain that a laboratory could maintain the precision required to have a 90% power to detect a five-fold difference in RNA copy number between two samples in the same batch. To achieve this goal, the QAP required an intra-assay standard deviation of no greater than 0.15 log10 RNA copies/mL of plasma. Panels for proficiency testing consisted of coded replicate samples and a common set of standards. Currently, 70 laboratories have participated in the program and have used both commercial and in-house assays. We demonstrated that at least 65-75% of the laboratories were capable of attaining the necessary level of intra-assay precision during the first two trials. The fitted regressions indicated the differences among laboratories that used the same kits were generally greater than the differences among population-average regressions for the kits themselves (8).

The use of an external QAP and a common set of standards reduced differences both among laboratories that used the same kit and among laboratories that used different kits. The variability of kit performance was minimal. Thus, use of a common set of standards across clinical trial protocols would allow for cross-protocol comparisons.

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