Quantification of HIV-1 RNA Using a Homogeneous Single-Tube Assay, Rajesh Patel,1 Rinie van Beuningen,2 Dianne van Strijp,3 and Nurith Kurn1 (1 Dade Behring Inc., San Jose, CA 95135; 2 Organon Teknika, NL 5281 RM Boxtel, The Netherlands; 3 address correspondence to this author at: Advanced Diagnostics Group, Dade Behring Inc., San Jose, CA 95135; fax 408-239-2707, e-mail Rajesh_Patel@dadebehring.com)

Quantification of HIV RNA has become an essential tool in the management of HIV infection and monitoring of gene expression. Although direct quantification of nucleic acids has been demonstrated, the principal method of quantifying nucleic acids still involves nucleic acid amplification and quantification of the specific amplification products. Most commonly, quantification of nucleic acids involves either real-time monitoring of the amplification products [with fluorescently labeled probes (TaqMan; Molecular Beacons)] or postamplification quantification [e.g., electrochemiluminescence (NuclisensTM)]. Real-time monitoring increases the dynamic range of quantification, but it also leads to more complex instrumentation, and the absence of an internal control in the reaction may lead to erroneous quantification because of sample interference. Quantitative nucleic acid sequence-based amplification (NASBA™) is carried out using internal controls added to the sample, but the presence of three internal controls and a postamplification electrochemiluminescence detection scheme makes this method susceptible to contamination of samples with previously amplified products.

We have combined highly sensitive NASBA with a highly sensitive chemiluminescent detection technology, Luminescent Oxygen Channeling Immunoassay (LOCI™), for homogeneous quantification of nucleic acids. NASBA is an isothermal exponential nucleic acid amplification system, based on the activity of avian myeloblastosis virus reverse transcriptase, RNase H, and T7 RNA polymerase, which predominantly produces a single-stranded RNA product. The specificity of HIV-1 amplification and detection is ensured by the use of specific primers and is further enhanced by the use of specific probe sequences for detection. The product concentration can be as high as 8.0 μmol/L after a typical NASBA reaction. Because the amplification product is single-stranded, there is no need to denature at the end of amplification for the binding of specific probes for either detection or quantification. Addition of an internal control (Qa), which bears sequence resemblance and is coamplified with the same primers as the wild type (WT), allows amplification of WT and Qa with equal efficiency. The ratio of the two amplicons at the end of amplification reflects the ratio of the two targets, WT and Qa, at the beginning of amplification. The two amplicons competitively bind to a common probe added to the amplification reaction. The distribution of the common probe between the WT and Qa amplicons is determined by LOCI. The ratio of the WT- and Qa-specific LOCI signals obtained from any NASBA/LOCI reaction reflects the ratio of the two amplicons present in the reaction. The WT analyte present in the sample can be easily determined from the ratio of the WT and Qa amplification products and the amount of Qa target added to the reaction.

LOCI is a highly sensitive homogeneous chemiluminescent assay involving a pair of ~200-nm latex bead particles: one dyed with an olefin (chemiluminescent particle) and the other dyed with a phthalocyanine photosensitizer dye (4). When excited by 680 nm light, the photosensitizer particle emits singlet oxygen (¹O₂) with a half-life of ~4 μs. Formation of analyte-specific bead pairs between the sensitizer and chemiluminescent particles allows an efficient transfer of ¹O₂ to the chemiluminescent particles, where it reacts with the olefin and generates a chemiluminescent signal with a half-life in the range of 0.5–30 s. In the absence of an analyte, the ¹O₂ decays in the medium without generating any chemiluminescent signal. In the present assay for nucleic acid detection and quantification, the LOCI particles are coated with specific oligonucleotide sequences that are capable of binding to their complementary sequences present on the probes.

Quantitative NASBA/LOCI is based on homogeneous and simultaneous detection of two analytes in a single tube. The two signals are obtained by using a pair of chemiluminescent particles, which were basically prepared as described by Ulman et al. (4) except that the first chemiluminescent particle was dyed with thiouene and 9,10-diphenyl anthracene (DPA) and the second particle was dyed with 2H-1,4-oxazine-3,4-dihydro-4,5-diphenyl-6-dimethylaminophenyl and Rubrene (N-phe). The DPA particle emits at ~400 nm with a half-life of ~1.0 s, and the N-phe particle emits ~560–590 nm with a half-life of ~30 s. The DPA particle binds to the WT-specific probe for the WT-specific HIV amplicon generated, whereas the N-phe particle binds to the Qa-specific probe (Fig. 1A). In addition to the specific probes, both amplicons compete for binding to a common probe added to the reaction, which in turn binds to a sensitizer bead. The trimolecular complex of the amplicon strand and the two probes hybridizes to oligonucleotides on the corresponding sensitizer and chemiluminescent latex particles. The ratio of the WT to Qa amplicons bound to the sensitizer particles is reflected by the ratio of the two LOCI signals generated in the assay. Detection of the two distinct chemiluminescent signals generated by the amplicon-bound bead pairs was carried out in a LOCI instrument (built in-house) that irradiates the sample tube with a 680 nm laser and collects the signal in two phases. The DPA emission is collected using a 395–415 nm bandpass filter. The N-phe signal is collected after the entire DPA signal has decayed, using a 550–650 nm bandpass filter. The DPA signal is corrected for the contributing N-phe signal by use of the known decay time constant of N-phe.

Initially, the HIV WT and Qa amplicons generated using the NASBA technology were quantified using the LOCI probes and beads. The LOCI signal was linear over 3 logs of amplicon concentrations. With LOCI, ~3 × 10⁶ amplicons could be detected in a 50-μL reaction with a
signal-to-background ratio of 1.7. The effect of LOCI reagents on NASBA was also assessed, and no inhibition of amplification efficiency was observed. LOCI reagents, consisting of the three LOCI oligonucleotide-coated particles and the three probes, were added to the NASBA Amplification kit (Organon Teknika), which included the HIV-specific primers. HIV target and Qa internal controls were added at appropriate concentrations, and amplification was carried out for 90 min as described in the package insert. The reaction tubes were then introduced into the LOCI reader, and the two chemiluminescent signals were collected as described. The WT signal was first obtained by irradiating the reaction tubes with a 680 nm laser for 1.0 s and then collecting the chemiluminescent signal for 0.5 s. This was repeated for a total of three times, after which the DPA signal was allowed to decay for 30 s before the Qa signal from the slow-decaying N-phe particle was collected for 10 s. The following algorithm was used to quantify the WT target present in the sample: Log HIV quantified (QT) = v[log[Qa/(WT + Qa)] + w + log[WT/(WT + Qa)], where WT and Qa are the corrected chemiluminescent LOCI signals. Ideally, the value of v is −1, and w is the log of Qa added to the reaction. However, v and w are derived from a fit-to-a-standard curve for a specific combination of probes, LOCI reagents, and assay conditions. Purified WT target and Qa were used to optimize the concentration of LOCI particles and the relevant probes for efficient quantification of the HIV target sequence.

This assay was used to quantify HIV-1 viral RNA (1 × 10^2 to 1 × 10^6 copies/mL) from plasma samples (Fig. 1B). HIV-1 plasma calibrators (Advanced Bioscience Laboratories) were used to prepare nucleic acid extracts based on Nuclisens extraction (Organon Teknika). The calibrator used is an HIV-1 producing cell line, which has been quantified with several technologies. The concentration of the HIV-1 calibrator used is equivalent to VQC (Red Cross Blood Banks, The Netherlands) and WHO standards.

The NASBA/LOCI detection limit of 320 copies per Nuclisens extraction converts into an amplification analytical sensitivity of 6 HIV-1 copies, based on the use of only 4% of the extract volume in the amplification and the typical nucleic acid recovery of 50% (data not shown).

Quantification was carried out using the NASBA/electrochemiluminescence (ECL) or the NASBA/LOCI method. The log of the average intra-experiment imprecision for NASBA/LOCI was 0.16 (n = 6 duplicates with input above 1 × 10^2 viral copies/mL). The accuracy of the assay, based on the use of known quantities of HIV-1 VQC calibrators, was 0.09 log (n = 6 duplicates) for NASBA/LOCI and 0.25 log for NASBA/ECL (n = 7 single measurements). The correlation between both quantification methods was 0.988.

The NASBA/LOCI assay procedure described is a simple method of nucleic acid quantification with a dynamic range of three orders of magnitude and a lower limit of detection of 1 × 10^3 HIV copies/mL. The system provides for containment and is easily amenable to automation. The single end-point readout allows simple instrumentation. In addition, LOCI provides an ultrasensitive homogeneous method for direct detection of nucleic acids with a limit of detection of 2 × 10^2 to 4 × 10^2 molecules.

References