Published in final edited form as:

Methods Mol Biol. 2009 ; 485: 55–72. doi:10.1007/978-1-59745-170-3_5.

Real-Time PCR Analysis of HIV-1 Replication Post-entry Events

Jean L. Mbisa^{#*}, Krista A. Delviks-Frankenberry^{#*}, James A. Thomas, Robert J. Gorelick, Vinay K. Pathak

[#] These authors contributed equally to this work.

Abstract

The reverse transcriptase enzyme plays an essential role in the HIV-1 life cycle by converting a singlestranded viral RNA genome into a double-stranded viral DNA through a complex process known as reverse transcription. The resulting double-stranded DNA is integrated into the host chromosome to form a provirus. A small proportion of the viral DNAs form dead-end circular products, which nevertheless can serve as useful surrogate markers for monitoring viral replication. Utilizing real-time PCR technology, it is possible to track and quantify different stages of the reverse transcription process, the proviruses, and the nonintegrated dead-end reverse transcription products.

Keywords

HIV-1; reverse transcription; real-time PCR; 1-LTR circle; 2-LTR circle; Alu-LTR real-time PCR

1. Introduction

Real-time PCR uses conventional PCR methods; however, the addition of a quenched probe that becomes fluorescent upon each PCR amplification step allows for direct quantification of the number of DNA copies initially present in the sample (1–4). The probe that is added to the PCR reaction (commonly referred to as the TaqMan probe), is an oligonucleotide that contains a quencher dye at the 3' end and a fluorescent reporter dye at the 5' end of the probe (4). Different fluorescent reporter dyes can be added to the 5' end, such as 6-carboxy-fluorescein (FAM), hexachloro-6-carboxy-fluorescein, or tetrachloro-6-carboxy-fluorescein, each of which emit light at different wavelengths. The quencher at the 3' end of the probe is typically 6-carboxy-tetramethyl-rhodamine (TAMRA). As shown in Fig. 5.1, a typical reaction starts when the probe binds to the target template during the cool-down phase after the melting step followed by binding of the forward and reverse primers (**Step 1**). At this stage, the quencher is in close proximity to the fluorescent reporter dye and therefore no fluorescence is emitted. The Taq polymerase that catalyzes the PCR reaction not only has a 5'–3' polymerase activity, but also a 5'–3' exonuclease activity (**Step 2**). Therefore, as the Taq polymerase synthesizes DNA, it cleaves and displaces the probe, releasing the quencher, and allowing fluorescence to be emitted (**Step 3**) before finishing each amplification cycle (**Step 4**). As a result, for each amplification cycle, the emitted fluorescence is recorded and

*These two authors contributed equally to this work.

the cycle number at which the fluorescence becomes detectable (cycle threshold; Q) is directly correlated to the number of templates in the reaction at that cycle. This correlation only holds true during the exponential phase of the PCR reaction. Utilizing a known copy number of input templates as a standard, a standard curve can be generated to determine the exact number of templates initially present in any unknown sample. Therefore, this technology can monitor, in real time, the progress of any PCR reaction and determine the exact DNA copy numbers per sample, with a high sensitivity that allows detection of 10 or fewer copies per sample (5). As an alternative to the TaqMan system, one can use SYBR Green I dye which intercalates into double-stranded DNA as it accumulates during the PCR reaction thereby producing a fluorescent signal that can be quantified (6–8). The benefits of the SYBR Green dye intercalation technique are that it is less expensive and can be universally applied to all PCR reactions. However, TaqMan probe chemistry is generally preferred over SYBR Green I dye chemistry because it provides a greater level of specificity. The TaqMan probe is specific for a given sequence whereas SYBR Green I dye binds all double-stranded DNA, including any PCR artifacts (primer-dimers or nonspecific amplification products), and therefore requires a final dissociation curve analysis to verify that the reactions are optimized so that the final amplified signal is specific to the target sequence (6–8).

The HIV-1 genome is initially plus-stranded RNA that must be converted into double-stranded DNA for the virus to complete its life cycle (9). The unique process by which the HIV-1 reverse transcriptase carries out DNA synthesis enables one to utilize specific primer and probe sets to track and quantify early, intermediate, and late events in reverse transcription (Fig. 5.2) (10–19). Early reverse transcription products can be monitored by using a primer/probe set that specifically amplifies minusstrand strong-stop DNA (Fig. 5.2A); minusstrand DNA transfer can be monitored by using a primer/probe set that flanks the U3-R-U5 junction (Fig. 5.2B); intermediate stages of reverse transcription during minus-strand DNA synthesis and elongation can be monitored with a primer/probe set specific to regions of the viral genome that are between the two LTRs, e.g., *gag* (Fig. 5.2C). Finally, plus-strand DNA transfer and late reverse transcription products can be monitored by a primer/probe set specific to the U5 and 5' untranslated regions of the viral DNA (Fig. 5.2D). Quantifying the final number of integrated proviruses into the host cell chromosome relies upon the presence of natural Alu repeats found throughout all human chromosomal DNA (11, 20, 21). A U5 specific forward primer and probe are used with a reverse primer that binds to the nearest Alu repeat, amplifying the integrated provirus (Fig. 5.2G). Every reverse transcription reaction does not always result in an integration event as some viral DNAs remain linear or circularize to form dead-end 1-LTR-and 2-LTR-circle products (Fig. 5.2E,F, respectively). The 2-LTR circular products can be quantified using primer/probe sets specific to U5 and U3, and the 1-LTR circle products can also be quantified using a specific primer/probe set which binds *gag* and *env* during real-time PCR (11,22–25).

The advent of real-time PCR has been instrumental to the field of retrovirology. Not only can real-time PCR be used for basic laboratory research, but it can also be used clinically to quantify HIV-1 viral loads and mutation frequencies in patients (5,26–30). Real-time PCR can be used to quantify the efficiency of reverse transcription and overall amount of viral DNA synthesis, quantify the levels of nonintegrated vs. integrated viral DNA for any

antiviral drug treatment, and determine the effects of an HIV-1 mutant or change in experimental conditions on viral DNA synthesis. Overall, real-time PCR technology has greatly enhanced our basic knowledge of HIV-1 post-entry events.

2. Materials

2.1. Cell Culture, Virus Production, Infections

1. 293T cells and/or a cell line transduced with an HIV-1-based vector. For example, a 293T cell line has been made that stably expresses the plasmid pHDV-eGFP (31) (named 293T-HIV-GFP cell line for remainder of text) (*see* Note 1).
2. An integration standard cell line generated by transducing cells with an HIV-1-based vector capable of a single round of replication (11,19) (*see* Note 2).
3. Dulbecco's Modified Eagle's Medium (DMEM) (Gibco/BRL, Bethesda, MD) supplemented with 10% fetal bovine serum (FBS; Hyclone, Ogden, UT), penicillin (50U/mL; Gibco/BRL) and streptomycin (50 µg/mL; Gibco/ BRL).
4. Plasmids: an HIV-1-based vector, e.g., pHDV-eGFP (31); pHCMV-G, a vector expressing the G glycoprotein of vesicular stomatitis virus (VSV-G) (32).
5. CalPhos Mammalian Transfection Kit (Clontech, Mountain View, CA).
6. Phosphate-buffered saline (PBS) without Ca^{2+} or Mg^{2+} (Cambrex, Walkersville, MD).
7. Millex GS 0.45-µm pore size filter (Nalgene, Rochester, NY).
8. HIV-1 p24 ELISA Kit (Perkin Elmer, Wellesley, MA).
9. DNase I (Roche, Indianapolis, IN).
10. Heat block set at 65 °C.
11. Hexadimethrine bromide (Polybrene), 1mg/mL stock (Sigma, St. Louis, MO).

2.2. DNA Isolation

1. QIAamp DNA Blood Mini Kit (Qiagen, Germantown, MD).
2. 2mL Collection tubes (Qiagen, Germantown, MD).
3. Dpn I restriction enzyme (NEB, Beverly, MA).

2.3. Real-Time PCR

1. A dedicated set of pipettes and filter tips for real-time PCR use only.
2. AmpliTaq Gold® DNA Polymerase, 5U/µL (PE-Applied Biosystems, Foster City, CA).
3. 100 mM dNTP Set, PCR grade, 4 × 25 µmol (Invitrogen, Carlsbad, CA).
4. 10X TaqMan® Buffer II (PE-Applied Biosystems).

5. 10X TaqMan® Buffer A (containing FAM-IO-ROX internal reference) (PE-Applied Biosystems).
6. 25mM MgCl₂ (PE-Applied Biosystems).
7. Forward and reverse primers (100 µM stocks).
8. Dual-labeled probe (100 µM stocks).
9. Plasmid DNA to be used as standard template as listed in Table 5.1 (*see* Note 3).
10. DNA from uninfected 293T cells.
11. PCR grade nuclease-free water (Ambion, Austin, TX).
12. MicroAmp® Optical 96-well Reaction Plates (PE-Applied Biosystems).
13. MicroAmp® Optical Caps for 96-well Reaction Plates (PE-Applied Biosystems).
14. Microfuge tubes (DNase/RNase free)
15. ABI7900HT (PE-Applied Biosystems) or equivalent Real-time PCR instrument.

3. Methods

The protocol outlined here describes the analysis of HIV-1 post-entry events during infection of cultured cells utilizing the TaqMan probe chemistry. In addition, this protocol can be easily modified to analyze other types of samples, for example clinical samples. To analyze post-entry steps during HIV-1 infection of cultured cells by real-time PCR, virus is produced by transfection of 293T cells with an HIV-1-based vector and an envelope protein expression construct, target cells are infected, and DNA is isolated from the infected cells.

3.1. Virus Production from Transfected Cell Lines

1. Plate 293T target cells in DMEM at density of 2.5×10^6 cells per 100-mm-diameter dish (*see* Note 1).
2. Twenty four hours later, replace medium with fresh DMEM. Transfect with 10 µg of pHDV-eGFP and 2 µg of pHCMV-G using CalPhos transfection kit following manufacturer's instructions (*see* Note 4).
3. Incubate the plates in 3% CO₂ incubator at 37 °C for 6 h.
4. Gently rinse the plates three times with 10 mL of PBS at room temperature supplemented with 1% FBS (v/v) (*see* Note 5). Add 10 mL of fresh DMEM media and incubate overnight in 5% CO₂ incubator at 37 °C.
5. Next morning, gently rinse the plates again three times with 10 mL PBS and add 10 mL of fresh DMEM media. Return to incubator.
6. Gently rinse the plates again 5–6 h later once with 10 mL PBS and add 10 mL of fresh DMEM media. Incubate overnight.

7. Harvest the virus containing supernatant the next morning and clarify the samples by centrifuging at $400 \times g$ for 4 min at 4 °C. Pass the supernatant through Millex GS 0.45 µm pore size filter.
8. Take an aliquot and perform p24 ELISA analysis following manufacturer's instructions.
9. Store the remaining sample at –80 °C until ready to use for infection of target cells.

3.2. Virus Production from Stable Cell Lines

1. Plate 293T-HIV-GFP cells at density of 2.5×10^6 cells per 100-mm-diameter dish (*see* Note 1).
2. Twenty four hours later, replace medium with fresh DMEM. Transfect with 2 µg of pHCMV-G using CalPhos transfection kit following manufacturer's instructions (*see* Note 4).
3. Incubate the plates in 3% CO₂ incubator at 37 °C for 6 h.
4. Remove the media and add 10 mL of fresh DMEM media.
5. Incubate the plates in 5% CO₂ incubator at 37 °C for 36–48 h.
6. Harvest the virus containing supernatant and clarify the samples by centrifuging at $400 \times g$ for 4 min at 4 °C. Pass the supernatant through Millex GS 0.45-µm pore size filter.
7. Take an aliquot and perform p24 ELISA analysis following manufacturer's instructions.
8. Store the remaining sample at – 80 ° C until ready to use for infection of target cells.

3.3. Infection

1. Plate 293T target cells at a density of 1×10^5 cells per 60-mm-diameter dish (*see* Note 1).
2. Twenty four hours later, infect target cells with p24-normalized virus as determined by HIV-1 p24 ELISA (*see* Note 6). Before infection, treat the virus with 10U/mL of DNase I plus 4mM MgCl₂ at 37 °C for 1 h to reduce transfected plasmid DNA carryover, which can contribute to the signal during real-time PCR.
3. For each virus, prepare in parallel a heat-inactivated control sample by incubating the virus at 65 °C for an hour to be used for determining carryover contamination from plasmid DNA.
4. Make the inoculum, for both live and heat-inactivated virus, by diluting so that p24 values are comparable, adjust final volume to 2mL per plate with DMEM, and add 5 µg/mL of Poly-brene.

5. Infect for 3 h with gentle rocking every 30–60 min.
6. Remove inoculum, gently rinse twice with PBS plus 1% FBS and add 4 mL of fresh DMEM.
7. Harvest total cellular DNA from both live virus- and heat-inactivated virus-infected cells at desired time points by removing the media and adding 1 mL of PBS. Scrape the plates or pipet vigorously to dislodge cells, and transfer cell suspension to 1.5 mL centrifuge tubes and spin at $1000 \times g$ for 4 min at 4 °C.
8. Discard supernatant and proceed to isolate DNA (see Section 3.4) or store cell pellets at –80 °C until ready to isolate DNA.

3.4. DNA Isolation

1. Resuspend cell pellets in 200 µL of PBS.
2. Proceed to extract the total cellular DNA following the protocol as stated in the Qiagen QIAamp DNA Blood Mini Kit.
3. Elute the DNA in 200 µL of elution buffer EB provided in the kit or nuclease-free water.
4. Digest DNA sample with *DpnI* restriction enzyme, which digests only plasmid DNA and therefore helps further to reduce plasmid DNA detection during real-time PCR. Use 0.1 U/µL in NEB Buffer 2 for 2–4 h at 37 °C.

3.5. Real-Time PCR Analysis of Postentry Reverse Transcription Steps

1. Analysis of the different stages of reverse transcription is normally carried out by completing a time course from 0 to 6 or 0 to 12 h, and subsequently comparing the different levels of reverse transcription products as time progresses. For example, collect and process samples at 0, 0.5, 1, 3, 6 and 12h postinfection.
2. If possible, use a UV-decontaminated hood when setting up all PCR reactions to avoid any cross contamination. Only use pipettes, RNase and DNase-free Eppendorf tubes and sterile PCR racks that are dedicated for real-time PCR use. All prepared reaction mixtures should be kept on ice.
3. Select the primer/probe set to use from the list in Table 5.1 depending on the stage of reverse transcription to be analyzed, i.e., RU5 for initiation of reverse transcription or early RT products, U3U5 products for minus-strand DNA transfer, *gag* for late minus-strand DNA synthesis, and U5Ψ for plus-strand DNA transfer and late RT products (see Note 7). PBGD or CCR5 primer/probe sets target cellular genes and are run for each sample to normalize for DNA recovery and the number of cells loaded in each reaction. The sequences of the primers and probes are listed in Table 5.2.
4. Prepare a standard curve from the appropriate standard template listed in Table 5.1 to be used to compute the copy numbers in each sample. Make a stock of 10^9 DNA copies/µL and dilute the working stock solution to 10^8 copies/µL. Prepare 11 dilutions for the standard curve: the first dilution should be made at a final

concentration of 10^7 copies per 10 μ L. Starting with the first dilution, make 10 additional threefold serial dilutions down to 169 copies per 10 μ L.

5. In a nuclease-free tube on ice, prepare a PCR master mix with all of the PCR reagents except the DNA as outlined in Table 5.3 (*see* Note 8). The reaction setup shown in Table 5.3 is for a 50- μ L reaction, but prepare enough PCR master mix for all the samples and standards in the experiment as well as a “no template” control (NTC). Include a 5% overage to compensate for pipetting error. Mix well and centrifuge briefly. It is recommended that all samples should be run in duplicate or triplicate to increase the accuracy of results.
6. Dispense 40 μ L of PCR master mix into each well of a MicroAmp® Optical 96-well Reaction Plate and add 10 μ L of DNA isolated from step 3.4 (100–500 ng), or 10 μ L of the serial dilution of standard DNA template or 10 μ L of water for the NTC. You may also run a blank containing 50 μ L of water. Securely cover all the wells (including unused ones) with the MicroAmp® Optical Caps for 96-well Reaction Plates. Mix well and centrifuge briefly to ensure that the entire sample is at the bottom of the well.
7. Run the PCR reaction using the thermocycling parameters outlined in Table 5.4 for analysis of different stages of reverse transcription or for the cellular gene controls, using an ABI Prism 7900HT sequence detection system (*see* Note 9).
8. After the reaction is complete, analyze the results according to the manufacturer’s instructions, especially paying great attention to the baseline and threshold settings (*see* <http://www3.appliedbiosystems.com/cms/groups/mcb-marketing/documents/generaldocuments/cms-042502.pdf> and <https://www2.appliedbiosystems.com/support/apptech/#rtPCR>). The blank and NTC samples should not give any signal. The viral standard curve will be used to calculate the copy number for each unknown sample. A graph plot showing a typical standard curve with values from live and heat-inactivated virus samples is presented in Fig. 5.3. The cellular gene copy numbers can be used to normalize for the number of the cells loaded in each reaction. A good standard curve should have a *y*-intercept of approximately 39–41 cycles and a slope between –3.321 and –3.5, which corresponds to an efficiency of 100–93%, respectively.

3.6. Real-Time PCR Analysis of 2-LTR-Circle Formation

1. Analysis of 2-LTR-circle formation is normally done using DNA collected 24 h after infection.
2. Select a primer/probe set from the list in Table 5.1 depending on whether you want to analyze the total number of 2-LTR circles (2-LTR TOT) or 2-LTR circles with complete junctions only (2LTR FJ). Also run a duplicate plate with PBGD or CCR5 primer/probe set for each sample to normalize for the number of cells loaded in each reaction.

3. Prepare a standard curve from the appropriate standard template listed in Table 5.1, to be used to compute the copy numbers in each sample as described in point 3 of Section 3.5.
4. Prepare the PCR master mix as described in point 4 of Section 3.5.
5. Dispense the PCR master mix and DNA into each well of a MicroAmp® Optical 96-well Reaction Plate as described in point 5 of Section 3.5.
6. Run the PCR reaction using the thermocycling parameters outlined in Table 5.4 for analysis of 2-LTR circles or for the cellular gene controls, using an ABI Prism 7900HT sequence detection system (*see* Note 9).
7. Analyze the data as described in point 8 of Section 3.5. The number of 2-LTR circles formed is normally expressed as a percentage of late RT products at 6 h after infection in a parallel infection. On average, during wild-type virus infection, 1–5% of late RT products at 6h ultimately form 2-LTR circles, depending on the infection target cell line (11,19).

3.7. Real-Time PCR Analysis of 1-LTR-Circle Formation

1. Analysis of 1-LTR-circle formation is normally done using DNA collected 24 h after infection.
2. Prepare a standard curve from the appropriate standard template listed in Table 5.1, to be used to compute the copy numbers in each sample as stated in point 3 of Section 3.5.
3. Prepare the PCR master mix as described in point 4 of Section 3.5 using the 1-LTRprimer/probe set (Table 5.1). Note the difference in the 1-LTRprimer and probe concentrations. Also run a duplicate plate with PBGD or CCR5 primer/probe set to normalize for the number of cells loaded for each reaction.
4. Dispense the PCR master mix and DNA into each well of a MicroAmp® Optical 96-well Reaction Plate as described in point 5 of Section 3.5.
5. Run the PCR reaction using the thermocycling parameters outlined in Table 5.4 for analysis of 1-LTR circle-formation or for the cellular gene controls, using an ABI Prism 7900HT sequence detection system (*see* Note 9).
6. Analyze the data as described in point 8 of Section 3.5. The number of 1-LTR circles formed is normally expressed as a percentage of late RT product at 6 h after infection determined from a parallel infection. On average, the proportion of 1-LTR:2-LTR circles formed during wild-type virus infection is 9:1 (11).

3.8. Alu-LTR Real-Time PCR Analysis of Viral Integration

1. Analysis of integrated proviruses is normally done using DNA collected 24–48 h after infection.
2. Prepare integration standard DNA from a 293T cell line transduced with an HIV-1-based vector as previously described (11,19) (*see* Note 2).

3. Determine the equivalent provirus copy number by correlating the Alu-LTR signal with late RT product using U5Ψ primer/probe set as previously described (11,20,21) (*see* Note 10). It is important to make this determination in the presence of the amount of cell-extract that will be present when measuring the unknowns (*see* Note 11). Make single use aliquots of 10^5 – 10^6 provirus/μL and keep at $-80\text{ }^{\circ}\text{C}$ until needed. Keep the cellular DNA as concentrated as is practical.
4. Measure the quantities of either PBGD or CCR5 to determine the number of cells present in each reaction, and dilute if necessary to insure that 100–500 ng of DNA is present when quantifying provirus. Otherwise, the signal obtained cannot be equated with quantities on the standard curve.
5. Prepare dilutions for the standard curve: the first dilution should be made at a final concentration of approximately 10^6 copies per 10 μL. Starting with this dilution, prepare threefold serial dilutions down to approximately 1,000 copies per 10 μL. Each dilution should also contain 100–500 ng of DNA from uninfected 293T cells per 10 μL, as a control for cellular DNA in the sample (*see* Note 11).
6. Prepare the PCR master mix as described in point 4 of Section 3.5 using the Alu-LTR primer/probe set (Table 5.1). Note the difference in the Alu-LTR primer and probe concentrations.
7. Dispense the PCR master mix and DNA into each well of a MicroAmp® Optical 96-well Reaction Plate as described in point 5 of Section 3.5.
8. Run the PCR reaction using the thermocycling parameters outlined in Table 5.4 for analysis of integrated proviruses or for the cellular gene controls, using an ABI Prism 7900HT sequence detection system (*see* Note 9).
9. Analyze the data as described in point 8 of Section 3.5. The number of integrated proviruses is normally expressed as a percentage of late RT product at 6 h after infection determined from a parallel infection. On average, during wild-type virus infection, 5–20% of late RT products at 6 h eventually integrate into the host genome, depending on the infection target cell line (11).

4. Notes

1. Virus can be produced by transfection of other types of cells or from others type of cells transduced with an HIV-1-based vector. Similarly, infections can be carried out using other types of cells that can support infection of the HIV-1 virus.
2. It is critical to prepare integration standards in the cell line that will be infected.
3. Precise quantification of DNA standards is critical for competent determination of copy numbers. First, it is important that DNA plasmids used to measure reverse transcription progression have been linearized. Likewise, the standards used to measure LTR circles should be circular. Prior to dilution of the standard

curve, make sure that the plasmid is not contaminated with other DNA forms (gel purify if necessary), then measure $A_{260/280}$ to determine concentration and purity. Pure DNA in H_2O should have a ratio of ~ 1.85 . Measure the optical density (O.D.) of several dilutions to insure that the quantities determined using A_{260} correlate with the dilutions. Extra care taken at this step is never a waste of time.

4. The amount of pHDV-eGFP plasmid that can be used for transfection of a 100-mm-diameter dish can range from 2.5 to 20 μg with little effect on virus titer. The use of less plasmid DNA might help reduce plasmid DNA carryover. Do not use more than 2 μg of pCMV-G because it is cytotoxic.
5. The rinsing of transfected or infected 293T cells should be done gently because the cells can peel off the plate easily. Use pipette dispenser on slow mode, mark a single area on plate for aspirating and dispensing fluid, wash maximum of six plates at a time using a pipette capable of holding 60 mL, gently rock back and forth, and side to side 10 times each. The number of washes can be reduced if there is concern about loss of cells.
6. Normally, 150–300 ng of unconcentrated virus inoculum is sufficient to give an infection rate of approximately 40–80% GFP-positive cells by FACS analysis.
7. To analyze minus-strand DNA transfer, compare the proportion of U3U5 products to RU5 products. For plus-strand DNA transfer compare the proportion of U5Ψ products to gag products. Once conditions have been worked out for a particular system, it is helpful to reduce the number of freeze–thaws between analyses, i.e., try to quantify vDNA species all at the same time rather than looking at RU5, then freeze, then look at U3U5, then freeze, then look at gag, etc. It is very important to measure quantities of DNA that will be directly compared (late/early) with the same type of instrument. For instance, if the lab has a Stratagene and an ABI instrument, it may cause problems to look at the early product on the Stratagene and the late product on the ABI machine.
8. There are commercially available PCRmaster mixes, e.g., Taq-Man Universal PCR Master Mix (PE-Applied Biosystems), that contain all the PCR reagents in a single tube except for the primers, probe and DNA. Other Taq polymerases can also be substituted for the AmpliTaq Gold polymerase. However, the use of other reagents might require optimization, especially for the Alu-LTR assay.
9. The thermocycling parameters given in this protocol are optimized for the ABI Prism 7700 sequence detection system. If the ABI Prism 7900HT is being used, the reactions can be run using the ABI Prism 7700 emulation mode. The thermocycling parameters may need to be adjusted to give optimal results when using other instruments. For example, the block temperature does not always correlate well with the sample temperature so that the machine will indicate that the block is at the right temperature, but the samples exhibit a lag. In addition, different blocks heat and cool at different rates. As an example of this type of optimization, when looking at CCR5, the ABI 7700 parameters are 95 °C 15",

55 °C 30", 60 °C 30". When using the Stratagene 4000 ×, the parameters are changed to 94 °C 30", 55 °C 37", 60 °C 30". In addition, the ramp rate for heating is altered to 1.5 °C/s from the default of 2.0 °C/s.

10. Verify that the HIV-1-based vector used to transduce the cells to make the integration standard has the Ψ region and sequence complementary to the reverse primer (MH532) of the U5Ψ primer/probe set. Otherwise design an alternate primer or use another primer/probe set.
11. Addition of DNA from uninfected cells is crucial for accurate quantification using Alu-LTR assay. For LTR circles and reverse transcription intermediate, extra cellular DNA does not have much effect because the primers and probes are specific for vDNA. But, because the Alu primer binds cellular DNA targets, the background DNA will sequester the Alu primer thereby decreasing the sensitivity and accuracy of the Alu-LTR reaction.

Acknowledgements

This research was supported in part by the Intramural Research Program of the NIH, National Cancer Institute, Center for Cancer Research. This project has been funded in whole or in part with federal funds from the National Cancer Institute, National Institutes of Health, under contract N01-CO-12400. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government.

References

1. Heid CA, Stevens J., Livak KJ, et al. (1996) Real time quantitative PCR. *Genome Res* 6, 986–994. [PubMed: 8908518]
2. Lie YS, Petropoulos CJ (1998) Advances in quantitative PCR technology: 5' nuclease assays. *Curr Opin Biotechnol* 9, 43–48. [PubMed: 9503586]
3. Holland PM, Abramson RD, Watson R., et al. (1991) Detection of specific polymerase chain reaction product by utilizing the 5'–3' exonuclease activity of *Thermus aquaticus* DNA polymerase. *Proc Natl Acad Sci USA* 88, 7276–7280. [PubMed: 1871133]
4. Livak KJ, Flood SJ, Marmaro J., et al. (1995) Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridization. *PCR Methods Appl* 4, 357–362. [PubMed: 7580930]
5. Palmer S., Wiegand AP, Maldarelli F., et al. (2003) New real-time reverse transcriptase-initiated PCR assay with single-copy sensitivity for human immunodeficiency virus type 1 RNA in plasma. *J Clin Microbiol* 41, 4531–4536. [PubMed: 14532178]
6. Gibellini D., Vitone F., Schiavone P., et al. (2004) Quantitative detection of human immunodeficiency virus type 1 (HIV-1) proviral DNA in peripheral blood mononuclear cells by SYBR green real-time PCR technique. *J Clin Virol* 29, 282–289. [PubMed: 15018857]
7. Gibellini D., Vitone F., Gori E., et al. (2004) Quantitative detection of human immunodeficiency virus type 1 (HIV-1) viral load by SYBR green real-time RT-PCR technique in HIV-1 seropositive patients. *J Virol Methods* 115, 183–189 [PubMed: 14667534]
8. Morrison TB, Weis JJ, Wittwer CT (1998) Quantification of low-copy transcripts by continuous SYBR Green I monitoring during amplification. *Biotechniques* 24, 954–958, 960, 962. [PubMed: 9631186]
9. Coffin JM, Hughes SH, Varmus H. (1997) *Retroviruses*, Cold Spring Harbor Laboratory Press, Plainview, N.Y.
10. Voronin YA, Pathak VK (2004) Frequent dual initiation in human immunodeficiency virus-based vectors containing two primerbinding sites: a quantitative in vivo assay for function of initiation complexes. *J Virol* 78, 5402–5413. [PubMed: 15113919]

11. Butler SL, Hansen MS, Bushman FD (2001) A quantitative assay for HIV DNA integration in vivo. *Nat Med* 7, 631–634. [PubMed: 11329067]
12. Karageorgos L., Li P., Burrell CJ (1995) Stepwise analysis of reverse transcription in a cell-to-cell human immunodeficiency virus infection model: kinetics and implications. *J Gen Virol* 76 (Pt 7), 1675–1686. [PubMed: 9049373]
13. Huber HE, McCoy JM, Sehra JS, et al. (1989) Human immunodeficiency virus 1 reverse transcriptase. Template binding, processivity, strand displacement synthesis, and template switching. *J Biol Chem* 264, 4669–4678. [PubMed: 2466838]
14. Majumdar C., Abbotts J., Broder S., et al. (1988) Studies on the mechanism of human immunodeficiency virus reverse transcriptase. Steady-state kinetics, processivity, and polynucleotide inhibition. *J Biol Chem* 263, 15657–15665. [PubMed: 2459125]
15. Victoria JG, Lee DJ, McDougall BR, et al. (2003) Replication kinetics for divergent type 1 human immunodeficiency viruses using quantitative SYBR green I real-time polymerase chain reaction. *AIDS Res Hum Retroviruses* 19, 865–874. [PubMed: 14585218]
16. Munk C., Brandt SM, Lucero G., et al. (2002) A dominant block to HIV-1 replication at reverse transcription in simian cells. *Proc Natl Acad Sci USA* 99, 13843–13848. [PubMed: 12368468]
17. Reardon JE (1993) Human immunodeficiency virus reverse transcriptase. A kinetic analysis of RNA-dependent and DNA-dependent DNA polymerization. *J Biol Chem* 268, 8743–8751. [PubMed: 7682554]
18. Buckman JS, Bosche WJ, Gorelick RJ (2003) Human immunodeficiency virus type 1 nucleocapsid p7⁺ fingers are required for efficient reverse transcription, initial integration processes, and protection of newly synthesized viral DNA. *J Virol* 77, 1469–1480. [PubMed: 12502862]
19. Thomas JA, Gagliardi TD, Alvord WG, et al. (2006) Human immunodeficiency virus type 1 nucleocapsid zinc-finger mutations cause defects in reverse transcription and integration. *Virology* 353, 41–51. [PubMed: 16784767]
20. Brussel A., Sonigo P. (2003) Analysis of early human immunodeficiency virus type 1 DNA synthesis by use of a new sensitive assay for quantifying integrated provirus. *J Virol* 77, 10119–10124. [PubMed: 12941923]
21. O'Doherty U., Swiggard WJ, Jeyakumar D., et al. (2002) A sensitive, quantitative assay for human immunodeficiency virus type 1 integration. *J Virol* 76, 10942–10950. [PubMed: 12368337]
22. Svarovskaia ES, Barr R., Zhang X., et al. (2004) Azido-containing diketo acid derivatives inhibit human immunodeficiency virus type 1 integrase in vivo and influence the frequency of deletions at two-long-terminal-repeat-circle junctions. *J Virol* 78, 3210–3222. [PubMed: 15016842]
23. Lusi EA, Guarascio P., Presutti C., et al. (2005) One-step nested PCR for detection of 2 LTR circles in PBMCs of HIV-1 infected patients with no detectable plasma HIV RNA. *J Virol Meth* 125, 11–13.
24. Gillim-Ross L., Cara A., Klotman ME (2005) HIV-1 extrachromosomal 2-LTR circular DNA is long-lived in human macrophages. *Viral Immunol* 18, 190–196. [PubMed: 15802963]
25. Jacque JM, Stevenson M. (2006) The inner-nuclear-envelope protein emerlin regulates HIV-1 infectivity. *Nature* 441, 641–645. [PubMed: 16680152]
26. Lewin SR, Vesanen M., Kostrikis L., et al. (1999) Use of real-time PCR and molecular beacons to detect virus replication in human immunodeficiency virus type 1-infected individuals on prolonged effective antiretroviral therapy. *J Virol* 73, 6099–6103. [PubMed: 10364365]
27. Halvas EK, Aldrovandi GM, Balfe P., et al. (2006) Blinded, multicenter comparison of methods to detect a drug-resistant mutant of human immunodeficiency virus type 1 at low frequency. *J Clin Microbiol* 44, 2612–2614. [PubMed: 16825395]
28. Mulder J., McKinney N., Christopherson C., et al. (1994) Rapid and simple PCR assay for quantitation of human immunodeficiency virus type 1 RNA in plasma: application to acute retroviral infection. *J Clin Microbiol* 32, 292–300. [PubMed: 8150937]
29. Piatak M Jr., Luk KC, Williams B., et al. (1993) Quantitative competitive polymerase chain reaction for accurate quantitation of HIV DNA and RNA species. *Biotechniques* 14, 70–81. [PubMed: 8424881]
30. Piatak M Jr., Saag MS, Yang LC, et al. (1993) High levels of HIV-1 in plasma during all stages of infection determined by competitive PCR. *Science* 259, 1749–1754. [PubMed: 8096089]

31. Unutmaz D., KewalRamani VN, Marmon S., et al. (1999) Cytokine signals are sufficient for HIV-1 infection of resting human T lymphocytes. *J Exp Med* 189, 1735–1746. [PubMed: 10359577]
32. Yee JK, Miyanohara A., LaPorte P., et al. (1994) A general method for the generation of high-titer, pantropic retroviral vectors: highly efficient infection of primary hepatocytes. *Proc Natl Acad Sci USA* 91, 9564–9568. [PubMed: 7937806]

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 5.1.

PCR amplification using TaqMan probe chemistry. After probe and primers bind to the template (**Step 1**), Taq polymerase initiates synthesis, displaces and cleaves the probe (**Step 2 and 3**), which allows fluorescence to be emitted. Polymerization is completed after one cycle of PCR amplification (**Step 4**).

Fig. 5.2.

Schematic representation of the HIV-1 reverse transcription and integration. Real-time PCR (shaded gray boxes containing arrows) can be used to analyze and quantify early, intermediate, and late reverse transcription products as well as integrated and unintegrated (1- and 2-LTR circles) HIV-1 viral DNA (Stages A-G). Thin black lines, RNA; thick black lines, DNA; thick gray lines, chromosomal DNA; dashed lines, RNase H degradation; cloverleaf, tRNA.

Fig. 5.3. Typical real-time PCR standard curve with values from live and heat-inactivated virus samples.

Table 5.1

Primer/probe sets for analysis of different stages of reverse transcription, 1- and 2-LTR circle formation, integrated proviruses, and cellular genes

Stage of reverse transcription ^a	Target sequence	Forward primer	Reverse primer	Probe	Standard template
A	RU5	hRU5-F2	hRU5-R	hRU5-P	pHDV-eGFP
B	U3U5	FST-F1	SS-R4	P-HUS-SS1	pHDV-eGFP
C	Gag	GagF1	GagR1	P-HUS-103	pHDV-eGFP
D	U5Ψ	MH531	MH532	LRT-P	pHDV-eGFP
E	1-LTR	LA1	LA15	1-LTR-P	pCR-1LTR
F	2-LTR FJ	SS-F4	LTR-R5	P-HUS-SS1	pJB1041
F	2-LTR TOT	SS-F4	LTR-R3	P-HUS-SS1	pJB1041
G	Alu-LTR	MH535	SB704	P-HUS-SS1	Special ^b
control	PBGD	nPBGD-For	nPBGD-Rev	nPBGD-Pr	pJB10S7
control	CCR5	CCR5-For	CCR5-Rev	P-CCR5-01	pRB2569 (19)

^aStages labeled in Fig. 5.2.

^bStandard for the Alu-LTR assay is the Integration Standard cell line, prepared as described in Section 3.8.

Table 5.2

Sequences of oligonucleotide primers and probes

Name of oligonucleotide ^a	Sequence
hRU5-F2 ^b	5'-GCCTCAATAAAGCTTGCCTTGA-3'
hRU5-R ^b	5'-TGACTAAAAGGGTCTGAGGGATCT-3'
hRU5-P ^b	5'-FAM-AGAGTCACACAACAGACGGGCACACACTA-TAMRA-3'
FST-F1 ^b	5'-GAGCCCTCAGATGCTGCATAT-3'
SS-R4 ^b	5'-CCACACTGACTAAAAGGGTCTGAG-3'
P-HUS-SS1 ^b	5' - FAM-TAGTGTGTGCCCCGTCTGTTGTGTGAC-TAMRA-3'
GagF1 ^b	5' -CTAGAACGATTTCGAGTTAATCCT-3'
GagR1 ^b	5' -CTAT CCTTTGAT GCACACAATAGAG-3'
P-HUS-103 ^b	5' -FAM-CATCAGAAGGCTGTAGACAAATACTGGGA-TAMRA-3'
MH531 ^b	5'-TGTGTGCCCCGTCTGTTGTGT-3'
MH532 ^b	5'-GAGTCCTGCGTCGAGAGATC-3'
LRT-P ^b	5'-FAM-CAGTGGCGCCCGAACAGGGA-TAMRA-3'
SS-F4 ^b	5' - TGGTTAGACCAGATCTGAGCCT-3'
LTR-R3 ^b	5' -AGGTAGCCTTGTGTGTGGTAGAT CC-3'
LTR-R5 ^b	5' - GTGAATTAGCCCTTCCAGTACTGC-3'
LA1 ^b	5'-GCGCTTCAGCAAGCCGAGTCCT -3'
LA15 ^b	5'-CACACCTCAGGTACCTTTAAGA -3'
1-LTR-P ^b	5'-FAM-AGTGGCGAGCCCTCAGATGCTGC-TAMRA-3'
MH535 ^b	5' -AACTAGGGAACCCACTGCTTAAG-3'
SB704 ^c	5' -TGCTGGGATTACAGGCGTGAG-3'
nPBGD-For ^c	5'-AGGGATTCACTCAGGCTCTTTCT-3'
nPBGD-Rev ^c	5' -GCATGTTCAAGCTCCTTGGTAA-3'
nPBGD-Pt ^c	5'-FAM-TCCGGCAGATTGGAGAGAAAAGCCTG-TAMRA-3'
CCR5-For ^c	5' -CCAGAAGAGCTGAGACATCCG-3'
CCR5-Rev ^c	5' -GCCAAGCAGCTGAGAGGTTACT-3'
P-CCR5-01 ^c	5'-FAM-TCCCCTACAAGAACTCTCCCCGG-TAMRA-3'

^a See Table 5.1 for the target sequence detected by each primer/probe set.^b Primer sequences were designed to amplify NL4-3 viral DNA, or vectors containing these sequences. Because of sequence variation between HIV-1 isolates, it may be necessary to modify or redesign the sequences.

^cPrimer sequences are designed for human genes. The use of cells from other species will require modification or redesign of these sequences.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Table 5.3

Setup of real-time PCR reactions

Amount	Component	Final concentration
3 µL	10X Buffer A	
2 µL	10X PCR Buffer II	1X
8 µL	MgCl ₂ (25 mM)	4mM
0.4 µL	dNTPs (25 mM)	0.2mM each
0.3 µL ^{a,b}	Forward primer (100 µM)	600 nM ^{a,b}
0.3 µL ^{a,b}	Reverse primer (100 µM)	600 nM ^{a,b}
0.04 µL ^{a,b}	Probe (100 µM)	80nM ^{a,b}
0.25 µL	AmpliTaq Gold® (5 U/µL)	1.25 U
10 µL	DNA (100 to 500 ng)	
to 50 µL	PCR Grade Nuclease-free Water	

^aFor the reaction to analyze 1-LTR circle formation, the forward and reverse primers are used at 400 nM, the probe is used at 200 nM (25).

^bFor Alu-LTR reactions, the forward primer is used at 50 nM, the reverse primer is used at 900 nM, the probe is used at 100 nM (*see* (19), Bushman website, <http://microb230.med.upenn.edu/>).

Table 5.4

Thermocycling parameters for real-time PCR reactions

Target of amplification	Cycling parameters		
	Step	Temperature	Duration
RU5, gag or U5Ψ	Denature ^a	95 °C	10 min
	45 PCR cycles		
	Denature	95 °C	15 s
	Anneal and extend	60 °C	1 min
PBGD or CCR5	Denature	95 °C	10 min
	45 PCR cycles		
	Denature	95 °C	15 s
	Anneal	55 °C	30s
Alu-LTR	Extend	60 °C	30s
	Denature	95 °C	10 min
	45 PCR cycles		
	Denature	95 °C	15 s
2-LTR circles	Anneal	60 °C	1 min
	Extend	72 °C	1 min
	Denature	95 °C	10 min
	45 PCR cycles		
1-LTR circles	Denature	95 °C	15 s
	Anneal and extend	66 °C	1 min
	Denature	95 °C	10 min
	45 PCR cycles		
	Denature	95 °C	30s
	Anneal	60 °C	30s
	Extend	72 °C	1 min
	Final extension	72 °C	5 min

^a AmpliTaq Gold (ABI) is acetylated to prevent DNA synthesis; 10min at 95 °C activates the polymerase. Platinum Taq (Invitrogen) is bound to an antibody to prevent DNA synthesis; 2 min at 95 °C is enough to activate the polymerase.