

Quantification of HIV-1 DNA

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[Abstract] The reverse transcription (RT) reaction is a critical step in HIV-1 life cycle. It is very strongly regulated and the target of several restriction factors (TRIM5 α , APOBECs, SAMHD1, etc.). The progress of reverse transcription can be followed by measuring viral DNA by quantitative PCR (qPCR). This method is sensitive enough to allow detection of low amounts of HIV-1 DNA in infected cells and discriminate between several types of reverse transcription intermediates (so called « early » and « late » RT products, 2 Long Terminal Repeat (LTR) circles, integrated DNA).

Materials and Reagents

1. Cells of interest, infected with HIV-1
2. Proteinase K (20 mg/ml) (>30 U/mg) (Eurobio, catalog number: GEXPRK01-B5)
3. AL buffer (QIAGEN, catalog number: 19075)
4. Phenol-chloroform – isoamyl alcohol (Sigma-Aldrich, catalog number: 77617-100ML)
5. Chloroform (Sigma-Aldrich)
6. Pure water (Life Technologies, Gibco®)
7. Absolute Ethanol (Sigma-Aldrich)
8. 3 M sodium acetate (pH 5.0)
9. DpnI Fast Digest restriction enzyme and reaction buffer (Fermentas, catalog number: FD1704)
10. Platinum SYBR Green qPCR Supermix UDG (Life Technologies, Invitrogen™, catalog number: 11733-046)
11. qPCR primers
12. Standard plasmids for HIV-1 (pNL4-3) and GAPDH (pGAPDH)

Equipment

1. Centrifuge
2. DNA-free hood
3. Speed-vac

4. Heat-seal machine
5. qPCR machine
6. 96-well qPCR plates (Eppendorf)
7. Plastic films for qPCR plates (Eppendorf)
8. Filter tips

Procedure

1. Collect at least 5×10^5 cells of interest infected with HIV-1, in 1.5 ml Eppendorf tubes and centrifuge for 3 min at $200 \times g$. Discard supernatant and wash pellets once in 1 ml of sterile PBS. Centrifuge for 3 min at $200 \times g$ and store dried pellets at $-80 \text{ }^\circ\text{C}$ up to a few months.
2. Open a new bottle of sterile PBS. Resuspend thoroughly the pellets in 180 μl of PBS. Add 20 μl (12 U) of Proteinase K and 200 μl of AL buffer. Vortex vigorously and incubate for 1 h at $56 \text{ }^\circ\text{C}$.
3. Add 400 μl of phenol-chloroform. Vortex vigorously and centrifuge 5 min at $16,200 \times g$. Collect the upper phase and add 400 μl of chloroform. Vortex vigorously and centrifuge 5 min at $16,200 \times g$.
Collect the upper phase, add 800 μl of absolute ethanol and 40 μl of sodium acetate 3 M (pH 5.0). Invert the tubes twice and incubate at $-20 \text{ }^\circ\text{C}$ for 1 h to allow DNA precipitation.
4. Centrifuge at least 30 min at $16,200 \times g$ in a cold centrifuge ($4 \text{ }^\circ\text{C}$). Discard the supernatant and wash with 500 μl of 70% ethanol. Discard the supernatant and dry the pellets for 5 min in the speed-vac.
5. Open a new bottle of pure water. Resuspend thoroughly the pellets in 20 μl of pure water and store DNA extracts at $-20 \text{ }^\circ\text{C}$ for up to a few weeks, or at $-80 \text{ }^\circ\text{C}$ for up to a few months.
6. Digest DNA extracts by the DpnI Fast Digest restriction enzyme to eliminate possible bacterial plasmid contamination. Add 4 μl of Fast Digest 10x buffer and 1 μl of the enzyme to the 5 μl of DNA extracts. Incubate at $37 \text{ }^\circ\text{C}$ for 15 min.
7. Incubate samples for 10 min at $95 \text{ }^\circ\text{C}$ to heat inactivate the enzyme, and to resolubilize DNA completely. Vortex each tube vigorously for at least 1 min.
8. Dilute a fraction of the sample in 40 volumes of pure water in order to prevent the Fast Digest Buffer from inhibiting the subsequent PCR reaction.
9. Under a DNA-free hood, prepare the reaction mix as follows (quantities per sample): 1 μl of each primer, 10 μl of SYBR Green and 3 μl of pure water. Distribute the reaction mix in the wells of the 96-well qPCR plate and avoid air bubbles. Outside the hood, add the 5 μl of the template DNA to quantify. As a negative control, add 5 μl of pure water instead of

- DNA. For an absolute quantification, use standards of known concentration (ranging from 10^3 to 10^7 copies, for instance). Each sample and each standard is quantified in duplicate. To quantify the « early » HIV products, use the following primers : GGC TAA CTA GGG AAC CCA CTG and GCT AGA GAT TTT CCA CAC TGA CTA A. to quantify the « late » RT products, use : GGC TAA CTA GGG AAC CCA CTG and CCT GCG TCG AGA GAG CTC CTC TGG. To quantify the amounts of cells, quantify the quantities of GAPDH with the following primers : GGG AAA CTG TGG CGT GAT and GGA GGA GTG GGT GTC GTT.
10. Cover the plate with an adhesive plastic film, centrifuge it briefly and heat-seal it.
 11. « Early » RT products (amplicon length: 183 bp) were quantified using the the following program: 35 cycles 10 sec 95 °C, 10 sec 57 °C, 15 sec 72 °C. For « late » RT products (amplicon length: 200 bp) and GAPDH (amplicon length : 303 bp), the PCR programm is : 35 cycles, 10 sec 95 °C, 15 sec 57 °C, 15 sec 72 °C).
 12. To convert the concentration (in $\mu\text{g}/\mu\text{l}$) of standard plasmids to the number of copies, use the following formula :
 To calculate the amount of HIV-1 DNA per cell, divide the absolute DNA of HIV-1 DNA copies per half the amount of GAPDH copies (because each cell contain two GAPDH copies).

$$\text{Number of copies} = \text{Concentration} / ((\text{size of the plasmid in bp} \cdot 660) / 6.02 \times 10^{17})$$
 13. A representative experiment of quantification of « early » and « late » RT products can be found in Figure 4D of Roesch *et al.* (2012).

Notes

1. All pipetting steps are performed with filter tips.
2. Prepare aliquots of pure water and store them at -20 °C. Use a new one for each experiment.
3. The « early » RT products are amplified in the 5' LTR region. The « late » RT products are amplified between the 5' LTR region and the *gag* gene. These regions are highly conserved, thus the indicated primers allow to quantify HIV-1 DNA of many different strains, such as NL4-3. To know if a particular strain will be amplified by these primers, one should align the sequence of the strain and of the primers and check that it is identical.
4. Unless stated otherwise, all steps are performed at room temperature.
5. It is not absolutely required to measure the DNA concentration of the different samples,

since the GAPDH normalization takes into account differences in DNA extraction efficiency.

Acknowledgments

This protocol is adapted from Roesch *et al.* (2012).

References

1. Roesch, F., Meziane, O., Kula, A., Nisole, S., Porrot, F., Anderson, I., Mammano, F., Fassati, A., Marcello, A., Benkirane, M. and Schwartz, O. (2012). [Hyperthermia stimulates HIV-1 replication](#). *PLoS Pathog* 8(7): e1002792.