Quantification of Retro- and Lentiviral Reverse Transcriptase Activity by Real-time PCR

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[Abstract] Quantification of retroviral reverse transcriptase activity in retrovirus containing supernatant by quantitative reverse transcription PCR as a method for titration of HIV, lenti- and retroviral vectors is described here. The procedure was optimized for use with LightCycler 480 (Roche, Vilvoorde, Belgium) and ABI 7300 real-time PCR system (reagents and procedures that are system specific will be marked accordingly in the protocol).

Materials and Reagents

1. MS2 RNA, aliquoted per 30 µl, storage -20 °C (F. Hoffmann-La Roche, catalog number: 10165948001)
2. Ribolock RNase inhibitor 40 U/µl, aliquoted per 10 µl, storage -20 °C (Fermentas, catalog number: EO0381)
3. HIV Reverse Transcriptase (Life Technologies, Ambion®, catalog number: AM2045)
4. Primers (directed against MS2 cDNA), storage -20 °C, (Eurogentec, Seraing, Belgium):
   FWD: 5'TCCTGCTCAACTTCCTGTCGAG3'
   REV: 5'CACAGGTCAAACCTCCTAGGAATG3'
5. Nuclease-free water, further referred to as H_2O (Life Technologies, catalog number: AM9939)
6. [Light Cycler 480]: LightCycler 480 SybrGreen I Master: Protect against light with aluminium foil (F. Hoffmann-La Roche, catalog number: 04707516001)
   OR [ABI 7300]: Eurogentec qPCR core kit for SYBR Green I (Eurogentec, catalog number: RT-QP73-05)
7. Tris (Sigma-Aldrich, catalog number: 154563-500G)
8. Ultrapure water (MilliQ water filtered with Milli-Q Gradient System) (Merck Millipore, Overijse, Belgium)
9. KCl (UCB, catalog number: 1592)
10. Glycerol (Merck Millipore, catalog number: 1.04094.1000)
11. Triton-X100 (MP Biomedicals, catalog number: 807423)
12. 2x Lysis buffer (Homemade), storage -20 °C (see Recipes)

13. Eurogentec qPCR core kit for SYBR Green I (see Recipes)

Equipment

1. Tissue Culture Plate 96W U-bottom (BD Biosciences, Falcon®, catalog number: 353077 or similar)
2. [Light Cycler 480]: LightCycler 480 Sealing foil (F. Hoffmann-La Roche, catalog number: 04729757001) OR [ABI 7300]: Optical adhesive film (Applied Biosystems, catalog number: 4311971)
3. [Light Cycler 480]: LightCycler 480 Multiwell plate 384 (F. Hoffmann-La Roche, catalog number: 04729749001) OR [ABI 7300]: MicroAmp Optical 96-well reaction plate with barcode (Applied Biosystems, catalog number: 4306737)
4. MicroAmp adhesive film applicator (Applied Biosystems, catalog number: 4333183)
5. Aluminium foil
6. Table top centrifuge

Procedure

Overview of the procedure:

- Preparations (see A): Make the lysis buffer / RNase inhibitor mix and qRT-PCR reaction mix.
- Lysis of the viral samples (see B): Addition of lysis buffer to the viral supernatant to release reverse transcriptase.
- qRT-PCR (see C): Addition of qRT-PCR reaction mix to the lysed samples, followed by the reverse transcription and quantitative PCR reaction.
- Analysis (see D): Calculation of the reverse trancritpase activity of the samples based on the obtained standard curve.

A. Preparations:

1. Calculate the amount of samples that will be included in the RT assay (x samples).
   Always include:
   a. Standard curve (SC) (containing 7 dilutions of viral supernatant or 7 dilutions of recombinant HIV reverse transcriptase + 1 medium control = 8 samples).
   b. 3 control viral supernatant (C) (= 3 samples)
   c. Your samples (S)
2. Make a mixture of 2x lysis buffer and RNase inhibitor (40 U/µl) (Table 1):

Table 1. Required Lysis buffer/RNase inhibitor

	Reagent per reaction (1 sample)	Reagent for all reactions ((x *1.25) samples)
2x lysis buffer	5 µl	5*(x*1.25) µl
RNase inhibitor (40 U/µl)	0.1 µl	0.1* (x*1.25) µl

Notes:

a. *Lysis buffer is very viscous. Therefore always make 25% extra and do not vortex to avoid formation of bubbles.*

b. *Keep RNase inhibitor on ice.*

3. Calculate the amount of samples that you will measure by qRT-PCR (n samples)

   Always include:

   a. Each sample in duplicate (= x*2 samples)

   b. H_2O twice (= 2 samples) (negative control of the qPCR)

4. Make the qRT-PCR reaction mix (see Table 2a [Light Cycler 480] or Table 2b [ABI 7300].):

   Notes:

   a. Make 10% extra.

   b. Keep LC 480 Sybr Green I mix, MS2 RNA and RNase inhibitor on ice.

   c. Do not vortex LC480 Sybr Green I mix.

   d. RNase inhibitor has to be diluted 10x in H_2O (final concentration: 4 U/µl).

Table 2a. qRT-PCR reaction mix for use on LightCycler 480 (384 well plates)

	Reagent per reaction (1 sample)	Reagent for all reactions ((n *1.1) samples)
LC 480 Sybr Green I (2x)	10 µl	10*(n*1.1) µl
Fwd primer (100 µM)	0.1 µl	0.1* (n*1.1) µl
Reverse primer (100 µM)	0.1 µl	0.1* (n*1.1) µl
MS2 RNA	0.1 µl	0.1* (n*1.1) µl
RNase inhibitor (10x diluted in H_2O, final concentration: 4 U/µl)	0.1 µl	0.1* (n*1.1) µl

Table 2b. qRT-PCR reaction mix for use on ABI 7300 real-time PCR system (96 well plates)

	1 sample	(n *1.1) samples
Eurogentec qPCR core kit for SYBR Green I	10.6 µl	10*(n*1.1) µl
Fwd primer (100 µM)	0.1 µl	0.1* (n*1.1) µl
Reverse primer (100 µM)	0.1 µl	0.1* (n*1.1) µl
MS2 RNA	0.1 µl	0.1* (n*1.1) µl
RNase inhibitor (10x diluted in H_2O, final concentration: 4 U/µl)	0.1 µl	0.1* (n*1.1) µl

B. Lysis of the viral samples

1. Add 5 µl of the viral supernatant or standard curve per well of a 96W U bottom plate.
2. Add 5 µl of Lysis buffer/RNase mix to each well and mix.
3. Incubate 10 min at room temperature (RT).
4. Add 90 µl nuclease-free water to each well and mix.
5. Spin plate for 3 min at 1,600 *x g* at RT in table top centrifuge.
6. Keep the plate on ice until transfer to the 384W [LightCycler 480] OR 96W [ABI 7300] qPCR plate.

C. qRT-PCR

1. Take a cooling element out of the -20 °C and cover the cooling element with aluminium foil. Put the 384W [LightCycler 480] OR 96W [ABI 7300] plate on the aluminium foil during the filling of the plate to prevent evaporation.
2. Put 10.4 µl [LightCycler 480] OR 11 µl [ABI 7300] of qRT-PCR reaction mix in each well of 384W [LightCycler 480] OR 96W [ABI 7300] plate.
3. Resuspend all the viral samples in the lysate plate.
4. Add 9.6 µl [LightCycler 480] OR 9 µl [ABI 7300] of sample to each well and resuspend.
5. Seal the plate using Sealing foil [LightCycler 480] OR Optical Adhesive film [ABI 7300], by removing the protective layer and press it to the plate using the applicator.
6. Spin the plate at 1,600 *x g* for 3 min at RT in table top centrifuge.
7. Run reaction:
   a. Light Cycler 480:
      Detection format: Sybr Green/HRM dye
      Reaction volume: 20 µl
      Program:
      Step 1: Reverse transcription (rt): 42 °C for 20 min.
      Step 2: Pre-incubation: 95 °C for 5 min.

Step 3: Amplification: 40 cycles of

95 °C for 5 sec

60 °C for 5 sec + detection

72 °C for 15 sec

Step 4: Melting curve

Step 5: Cooling

b. Run on the ABI Prism 7300:

Reaction volume: 20 µl

Program:

Step 1: Reverse transcription (rt): 42 °C for 20 min.

Step 2: Pre-incubation: 95 °C for 2 min.

Step 3: Amplification: 40 cycles of

95 °C for 5 sec

60 °C for 30 sec + detection

72 °C for 15 sec

Step 4: Melting curve

D. Analysis

1. Export the obtained Cq (Cycle of quantification) values from the LC480 or ABI Prism 7300.
2. Calculate for each sample the average Cq value of the duplicate measurements.
3. Make the standard curve: plot the average Cq value of SC1-SC7 versus the logarithm of the RT activity (expressed as mU RT/ml), determine the trendline and the formula expressing the correlation between the Cq values and the logarithm of the RT activity.
4. Use the obtained formula to calculate the absolute RT value of the samples.

Notes

Control samples:

1. Standard curve:

   For absolute quantification of retroviral RT activity one can measure a serial dilution with known concentration of recombinant reverse transcriptase in parallel with the samples of interest (see a). Alternatively, a standard curve can be made by using serial dilution of a retro- or lentiviral supernatant of choice. In the latter case, it necessary to determine the RT activity of this supernatant in a first experiment by running a recombinant reverse transcriptase standard curve in parallel. For later experiment the serial dilution of the retroviral supernatant can be used as a standard curve (see b).

a. Use recombinant HIV Reverse transcriptase as standard curve
   SC1 = solution of 200 mU/µl HIV Reverse Transcriptase. This is 1/50 dilution of the stock solution (10 U/µl)
   SC2-SC7 are made by serial 1/10 dilution of SC1 in cell culture medium (preferably the same medium in which retro- and lentiviruses were produced)
   Sample SC8 = cell culture medium (negative control)
b. Use high titer retroviral supernatant as a standard curve
   SC1 = high titer retro- or lentiviral supernatant of your choice.
   This sample can be any viral supernatant that is more concentrated than the samples being analysed. As mentioned above, the RT activity of this sample should be determined in an initial experiment by running a recombinant reverse transcriptase standard curve (see a) in parallel.
   SC2-SC7 are made by serial 1/10 dilution of SC1 in cell culture medium (preferably the same medium in which retro- and lentiviruses are produced)
   Sample SC8 = cell culture medium
   All samples are aliquoted per 8.5 µl in PCR strips. For each assay a new strip will be used.

2. Control samples C1, C2, C3:
   3 retroviral supernatants of your choice that will be measured in each RT assay for quality control: The obtained RT activity of the control samples should be similar over different RT assays and offers a control for interrun variation.

Recipes

1. 1 M Tris-HCl solution
   12.1 g Tris in 50 ml ultrapure water-adjust pH to 7.4 with HCl
   Adjust total volume to 100 ml with MilliQ water
2. 2x Lysis buffer (storage -20 °C)
   0.25% Triton X-100, 50 mM KCl, 100 mM Tris-HCl (pH 7.4), 40% glycerol
   Add 50 ml of 1 M Tris-HCl solution to a 500 ml bottle
   Add 1.86 g KCl to the 500 ml bottle
   Add 200 ml glycerol
   Add 1.25 ml of Triton-X100
   Mix the solution very well until everything is dissolved
   Add MilliQ water up to a final volume of 500 ml
   Aliquot 40 ml in 1.5 ml screw cap microtube
   Aliquot the remainder in 50 ml tubes

3. Eurogentec qPCR core kit for SYBR Green I (store aliquots at -20 °C)
   Composition of 10.6 µl:
   2 µl 10x reaction buffer
   1.4 µl of 50 mM $MgCl_2$
   0.8 µl of 5 mM dNTP mix
   0.1 µl of HotGoldS Tar Taq polymerase
   0.6 µl SYBR Green I
   5.7 µl of nuclease-free water

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