Quantification of Integrated HIV DNA by *Alu*-HIV PCR

Authors: Sofie Rutsaert, Eva Malatinkova, Pawel Bonczkowski, Maja Kiselinova, Ward De Spiegelaere

http://www.integratedhivpcr.ugent.be/

Quantification of integrated proviral HIV DNA by repetitive-sampling *Alu*-HIV PCR in patients.

MATERIALS

REAGENTS (**Note 1**)
DNA extraction kit (Qiagen DNeasy Blood & Tissue kit, cat. 69504/69506)
GoTaq® G2 DNA Polymerase (Promega, cat. M7845)
Nuclease-free water (Qiagen, cat. 129114)
dNTP mix (Promega, cat. U1511)
Primers (see Table 1, ordered at Integrated DNA Technologies)
LightCycler® 480 Probes Master, 2x concentrated (Roche Applied Science, cat. 04707494001)

EQUIPMENT
Thermal Cycler Applied Biosystems® 2720 (cat. 4359659)
LightCycler® 480 System II (Roche, cat. 05015278001)

Table 1: Primers used for *Alu*-HIV PCR

Primers	Sequence
1st PCR (Alu-Gag PCR)	
Forward primer *Alu*(1)	5'-GCCTCCCAAAGTGCTGGGATTACA-3'
Reverse primer *Gag*(1)	5'-GTTCCTGCTATGTCACTTCC-3'
2nd PCR (qPCR)	
Forward primer RU5 (R Forward)(2)	5'-TTAAGCCTCAATAAAGCTTGCC-3'
Reverse primer RU5 (U5 Reverse)(2)	5'-GTTCGGGCGCCACTGCTAGA-3'
Probe(3)	5'-CCAGAGTCACACAACAGACGGGCACA-3'

(1) Liszewski *et al.* 2009; (2) Yu *et al.* 2008 ; (2) Yun *et al.* 2002

PROCEDURE

Preparation

Isolate genomic DNA from the target cells using the DNA extraction kit. To estimate the volume of material used for the input in PCR reaction, one can either use a fixed number of input cell equivalents, e.g. 7500 equivalents per reaction as described by Liszewski et al (2009), or adjust input to the amount of total HIV DNA, as estimated by a prior total HIV DNA quantification by real time or digital PCR (**Note 2**).

Introductory information

1. Depending on the origin of the DNA for analysis (patient samples or cell cultures), the input for the 1st PCR need to be selected. For patient samples, the reaction works best if the input is in the range of 1-2 HIV DNA copies/well (**Note 3**). Primers used are denoted in Table 1.

2. To maximize the power of the analysis, 40 replicates for the *Alu-Gag* primers combination and minimally 20 for Gag only primer (background) are performed. If enough material is present, an equal number of Gag-only and *Alu-Gag* replicates is preferred (**Note 4**).

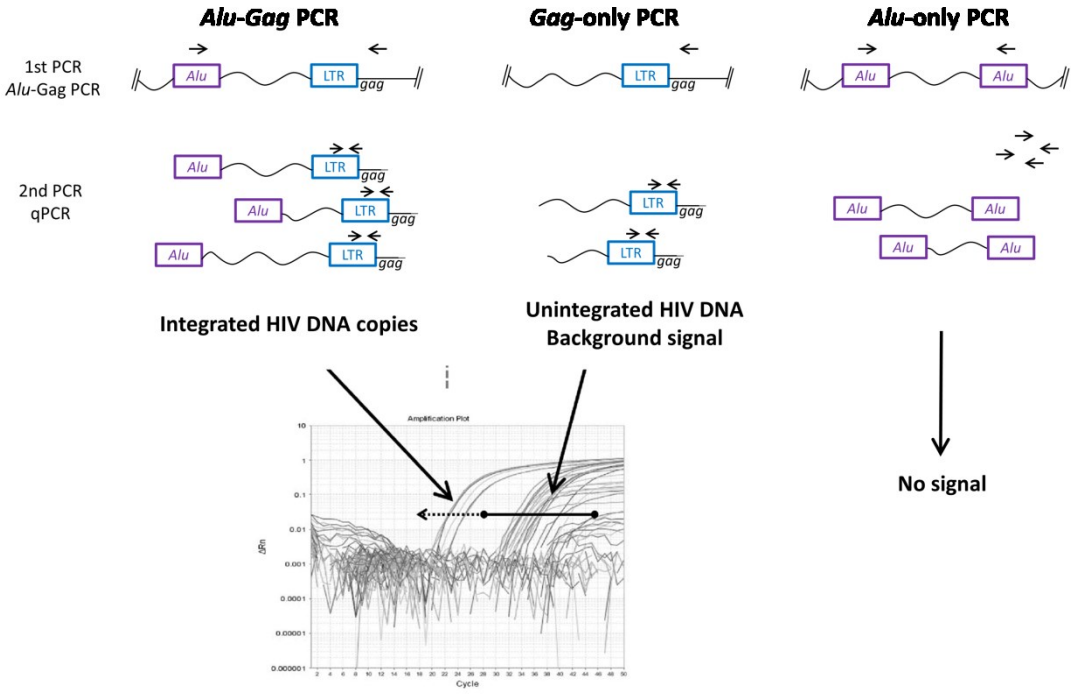

Figure 1: Principle of *Alu-Gag* HIV PCR, the *Alu-Gag* PCR is shown on the right, and initiates a logarithmic amplification of integrated HIV DNA only, the *Gag*-only PCR is required to estimate the background fluorescence which originates from a linear amplification in the first PCR (this may be expected in the *Alu-Gag* HIV PCR with unintegrated HIV DNA). Finally, an optional *Alu*-only PCR may be performed to ensure that no false positive reactions are generated.

1st PCR – *Alu*-Gag PCR

The PCR reaction

1. Prepare the mix for the appropriate number of replicates consisting of:

PROMEGA GoTaq	***Alu-gag* mix µl/reaction**	***Gag* only mix µl/reaction**
Buffer (5X)	4	4
dNTP's (10 mM)	0.4	0.4
H_2O	4	4.2
GoTaq	0.2	0.2
Forward primer (nM)	0.2	
Reverse primer (nM)	1.2	1.2

The total volume adds up to 10 µl per reaction.

2. Distribute the mix in a 96 well plate (10 µl per well) taking the amount of replicates of both *Alu-Gag* and *Gag* only into account, add 2 wells of each for the positive and negative controls.

3. Add 10 µl of the DNA solution to each well except for the controls. In the controls, add 9 µl of water and 1 µl of control DNA (from the integration standard) to the positive controls, and 10 µl of water to the negative controls.

4. Seal the plate and run the PCR protocol in the Thermal Cycler, using the following cycling conditions:

Reaction details	temperature (°C)		time
40X	initial denaturation	95	02:00
	denaturation	95	00:15
	annealing	50	00:15
	elongation	70	03:30
	pre-cooling step	70	00:15

2nd PCR - qPCR

1. The relative quantification is performed with a qPCR on the PCR product from the first PCR step

2. Prepare the qPCR mix consisting of:

	µl/reaction
Mastermix Roche	5
H2O	2
Probe (10 µM)	0.2
Forward primer (10 µM)	0.4
Reverse primer (10 µM)	0.4

3. Distribute the mix in a 384 well plate, 8 µl per well.

4. To each of the wells, add 2 µl of the 1st PCR solution from a corresponding well. Maintain the number of replicates, use each replicate only once.

5. Run the reaction on LightCycler® 480 System II (Roche).

6. The cycling conditions are:

Reaction details		temperature (°C)	time
	initial denaturation	95	05:00
	denaturation	95	00:15
45X	annealing	60	01:00
	cooling step	40	15:00

Data analysis

To assess the number of integrations per cell, there is an Excel template (Fig. 2) provided (http://www.integratedhivpcr.ugent.be/) in which the raw quantitative output of the *Alu*-HIV PCR should be inserted as described below (De Spiegelaere *et al* 2014).

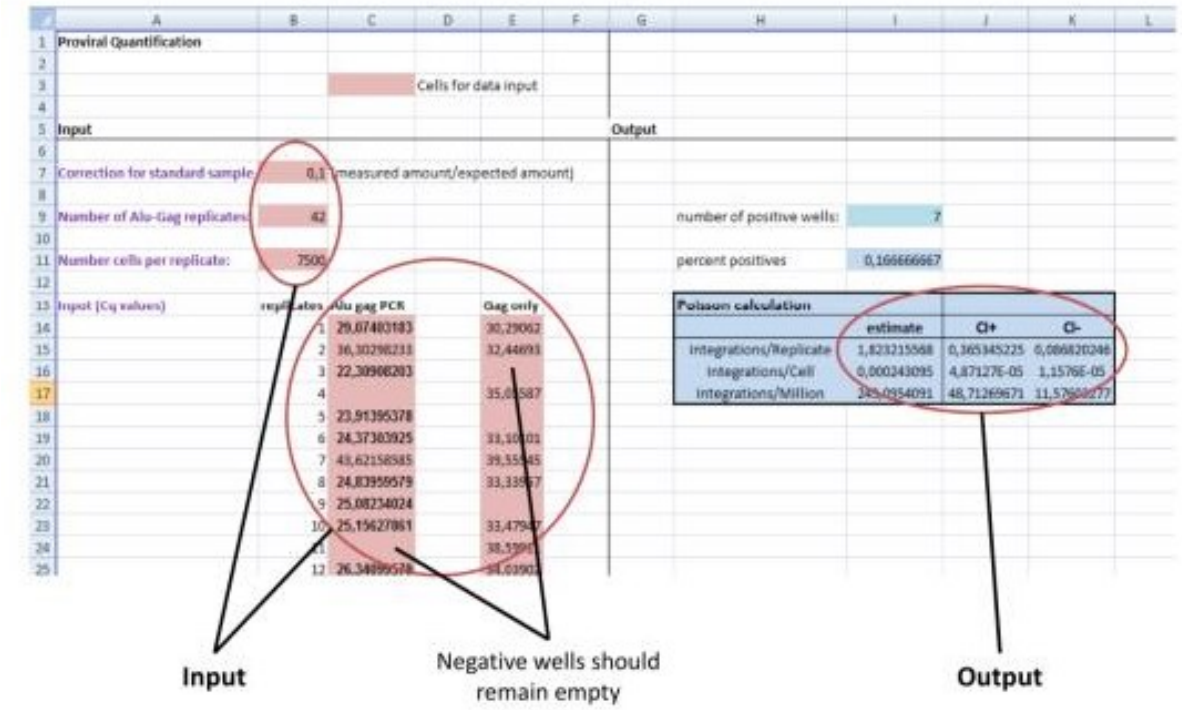

Figure 2: Excel template (De Spiegelaere *et al* 2014)

The excel template composed for *Alu*-HIV PCR quantification: the coloured cells in the lower left quadrant require data input, i.e. the correction factor as measured on the standard for calibration to assess the fold difference between the observed and expected number of integrations/well, the total number of *Alu*-HIV replicates (required for accurate error estimation) the number of cells per replicate reaction (assessed by a qPCR on a reference gene, e.g. RPP30), and the raw quantification cycle (Cq) values (note that the Cq values are in some real-time PCR programs referred to as treshold cycle (Ct), crossing point (Cp) or take-off point (TOP)) of the *Alu*-HIV PCR and the HIV only PCR. Note that negative reactions should be left empty not to interfere with the analysis. After data input, the results are available in the shaded areas of the lower right quadrant, showing the amount of proviral HIV per well, per cell or per million cells along with the confidence intervals of each estimate.

References

References

Liszewski, MK, Yu, JJ and O'Doherty, U (2009). Detecting HIV-1 integration by repetitive-sampling Alu-gag PCR. Methods 47:254-260.

De Spiegelaere, W, Malatinkova, E, Lynch, L, Van Nieuwerburgh, F, Messiaen, P, O'Doherty, U and Vandekerckhove, L (2014). Quantification of integrated HIV DNA by repetitive-sampling Alu-HIV PCR on the basis of poisson statistics. Clin Chem 60:886-895.

Yu, JJ, Wu, TL, Liszewski, MK, Dai, J, Swiggard, WJ, Baytop, C, Frank, I, Levine, BL, Yang, W, Theodosopoulos, T and O'Doherty, U (2008). A more precise HIV integration assay designed to detect small differences finds lower levels of integrated DNA in HAART treated patients. Virology 379: 78-86.

Yun, Z, Fredriksson, E and Sonnerborg, A (2002). Quantification of Human Immunodeficiency Virus Type 1 Proviral DNA by the TaqMan Real-Time PCR Assay. Journal of Clinical Microbiology 40:3883-3884.

Notes

Note 1

These reagents and materials are being used by us. An alternative method for *Alu*-HIV PCR has also been described by Liszewsky *et al* (2009)

Note 2

Using a fixed number of cell equivalents provides a standardized reaction. However, in patient samples with limited HIV DNA, most reactions become negative which may limit the quantitative power of the Poisson calculation afterwards. By estimating the number of HIV DNA templates before *Alu*-HIV PCR, the concentration of HIV DNA templates can be estimated to get an optimal Poisson quantification.

Note 3

We generally start with 10 DNA copies for cell cultures per reaction, considering the fact that cell culture generally have a higher concentration of unintegrated HIV DNA, compared to patient derived samples.

Note 4

We use a cut-off of five positive *Gag*-only PCR reactions to estimate the background. If this number is not reached, the amount of *Gag*-only PCR replicates should be augmented.