Lentivectors usually contain DNAs coding for proteins other than GFP or lacZ and promoters that are specifically active in various primary cells and tissue. Therefore, the majority of LVs will not be titrable on HCT116 cells by flow cytometry. An alternative method is thus needed to measure the number of copies of LV stably integrated in HCT116 target cells after transduction. This assay, based on qPCR, is unequivocal as it measures the number of LV DNA copies integrated in the target cell genome. However, the ultimate test of the functionality of the vector is in cells supporting the activity of the promoter driving the transgene, through techniques adapted to the nature of its product.

The QPCR assay described here requires a real time PCR machine, and proceeds as follows. HCT116 cells are transduced and the DNA is extracted using a genomic DNA extraction kit. A fraction of this DNA is then analyzed for copy number of HIV sequences using the real-time PCR protocol.

**NOTE:** Always use pipet tips containing aerosol-barrier filters when preparing solutions, mixes, samples, and plates for QPCR, to prevent cross-contamination.

**Materials**

HCT116 cells (ATCC cat. no. CCL-247).
Lentiviral vector (LV) sample for titration (see Basic Protocol) and DNA from positive and negative control cells (see annotation to step 1).
pAlb (available from Addgene, http://www.addgene.org) a pRRL vector in which the target sequence of the albumin primers used for normalization has been cloned. This plasmid allows performing a standard curve.
DNAeasy Genomic DNA Extraction Kit (Qiagen).
Kit for preparing QPCR master mix (TAQMAN UNIVERSAL PCR MASTR MIX, NO UNG/ Applied Biosystems (4324020), including 2× reaction buffer.
Primers and probe for Gag, WPRE and Albumin detection.

<table>
<thead>
<tr>
<th>Sequence detected</th>
<th>Primer/Probe name</th>
<th>Primer/Probe sequence</th>
<th>Probe fluorophores</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gag</td>
<td>Gag_forward</td>
<td>GGAAGCTAGAAAGATCCAGGTA</td>
<td>FAM-BHQ</td>
</tr>
<tr>
<td></td>
<td>Gag_reverse</td>
<td>GGTGTAAGCTGTCATCCAGTTGTC</td>
<td>FAM-BHQ</td>
</tr>
<tr>
<td></td>
<td>Gag_probe</td>
<td>ACGGCTCTTCAAGTCTTTCACCAGG</td>
<td>FAM-BHQ</td>
</tr>
<tr>
<td>WPRE</td>
<td>WPRE_forward</td>
<td>GCCACTGACATCCCTTGCGT</td>
<td>FAM-BHQ</td>
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<tr>
<td></td>
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<td>AGGGACGTTAGAAAGGAGCG</td>
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</tr>
<tr>
<td></td>
<td>WPRE_probe</td>
<td>ACCTCTTTCCATGCTGCTGCC</td>
<td>FAM-BHQ</td>
</tr>
<tr>
<td>Albumine</td>
<td>Alb_forward</td>
<td>GCTGCTATCTTCTTGGGCTGT</td>
<td>FAM-BHQ</td>
</tr>
<tr>
<td></td>
<td>Alb_reverse</td>
<td>ACTCATGGGAGCTGCTGTTTGC</td>
<td>FAM-BHQ</td>
</tr>
<tr>
<td></td>
<td>Alb_probe</td>
<td>CCTGCTCATGCCCAACAAATCTCTCC</td>
<td>FAM-BHQ</td>
</tr>
</tbody>
</table>

Gag oligos are used for amplification of HIV-1 derived vector sequences and are specific for the 5' end of the gag gene. This sequence is present in all HIV-1 vectors, as it is part of the extended packaging signal. WPRE oligos amplify the WPRE sequence present in almost all later generation LV vectors (see the commentary section). Alb oligos are used to normalize for the amount of genomic DNA and are specific for the human albumine gene.

Stocks of probes and primers usually come lyophilized and are diluted to 10 μM in water.
MicroAmp 96-well optical reaction plate (Applied Biosystems)
Optical adhesive Film (Applied Biosystems)
Centrifuge with microtiter plate carrier
Real-time PCR machine (7900HT Sequence Detector, Applied Biosystems)
Computer running SDS7900HT software (Applied Biosystems) and Microsoft Excel
Additional reagents and equipment for transducing HCT116 cells with lentivectors (Support Protocol 2)

Prepare DNA sample

1. Transduce HCT116 cells with the lentiviral vector of interest in 12-well tissue culture plates.

   For non concentrated vector transduce the cells with: 1000μl, 500μl, 100μl, 50μl and 20μl of crude supernatant. Except for the 1000μl point, complete the volume to 500μl with DMEM-10. Keep one well as non transduced control (NI). Perform each transduction in duplicate.

   For concentrated vector transduce cells with 4, 2, 1, 10⁻¹, 10⁻² μl of vector in 500μl of fresh DMEM-10.

   *The additional points with higher vector dose ensure an accurate quantification by QPCR.*

2. Wait at least 96h after transduction to reduce the contamination from plasmids DNA transfected possibly co-purified with the vector.

   *Cells containing known vector copy number of LV can be used as internal control. For this purpose HCT116 cells are transduced with a LV vector, subcloned as individual cells and analyzed by southern blot. Transduce cells with a high dose of LV to obtain cellular clones with high number of integration. Lower values can then be easily obtained by diluting the extracted gDNA with the one from non transduced HCT116.*

3. Extract target cell DNA from each individual well of the 12-well plate using a DNAeasy kit according to the manufacturer’s recommendations. For the DNA elution step, use 100 μl of AE buffer instead of 200 μl. Store DNA at –20°C until use.

Prepare plasmid standard curve

4. First adjust accurately the DNA concentration of pAlb to 1mg/ml. The pAlb concentration is 1,2,10¹¹ molecules/μl

   *Calculation of the molecule number is the following:*

   \[
   pAlb \text{ is a 7539 bp plasmid.} \\
   7539 \times 660 = 5.10^6 \text{ g} \leftrightarrow 1 \text{ mol} \\
   \text{if plasmid concentration is 1mg/ml then } 1.10^6 \text{ g} \leftrightarrow 2.10^{13} \text{ mol} \\
   2.10^{13} \text{ mol} \times 6.02.10^{23} = 1.2.10^{11} \text{ molecules/μl}
   \]

   The first point of the standard curve is 1.10⁷ molecules in 8μl = 1,25.10⁶ molecules/μl. The standard curve is then generated by serial 10-fold dilution until 10 molecules in 8 μl.

5. Dilute the plasmid as following:
Perform reaction

6. Prepare a mix (containing everything but the sample DNA) for the number of wells needed for the QPCR analysis, including all samples and standards in duplicates, according to the following recipe (9 μl per well):

- 8.5 μl 2× reaction buffer from Applied Biosystem QPCR MasterMix
- 0.17 μl forward primer (10μM)
- 0.17 μl reverse primer (10μM)
- 0.17 μl probe (10μM)

The Taq polymerase is part of the 2× reaction buffer.

7. Place 9 μl of this mix into each well of a 96-well optical reaction plate.

8. Add 8 μl sample DNA (from step 1 and from step 2) to each of the appropriate wells. DNA concentration of sample must be between 50 to 100 ng in 8 μl.


10. Centrifuge the plate 1 min at 200 × g, room temperature, to bring all liquid to the bottoms of the wells.

11. Place the 96-well plate in the real-time PCR machine and run the appropriate program depending on the fluorochromes (FAM) and quenchers (BHQ = non fluorescent ) used in the TaqMan probes.

The amplification cycles used on the 7900HT machine are:

- 10 minutes at 95°C
- 50 cycles
  - 15 seconds at 95°C
  - 1 minute at 60°
Be aware that the precise settings of a QPCR protocol depend on the real-time PCR machine and mix used. This aspect is beyond the scope of this protocol. If not familiar with QPCR techniques, one should seek advice from a local QPCR expert or from the technical support department of the supplier of the real-time PCR machine.

**Analyze results**

12. Analyze the amplification reactions using SDS7900HT software.

*Assign for each point of the standard curve in the set up of the SDS document the value corresponding and the standard status. The SDS program will automatically calculate the standard curve and the quantity of each unknown sample for each gene of interest.*

An example of amplification profiles of HIV sequences in human DNA is given in Figure 4.21.1 (as displayed by SDS).

13. Set the threshold values (Ct) where the amplification curve is the steepest, both for the gene of interest (Gag or WPRE) and for the internal control (Alb).

*These Ct values are the number of cycles required for the amplification curve to cross the absorbance threshold values.*

14. Export the results as a Microsoft Excel sheet.

15. Using Excel, calculate for each sample the HIV sequences copy number per cell using the following formula:

\[
\text{Vector copy number} = \frac{\text{Quantity mean of WPRE or Gag sequence}}{\text{Quantity mean of Alb sequence}} \times 2
\]

*The 2-fold factor reflects the fact that the Albumin sequence is present in two copies per genome (two alleles).*

16. Calculate the titers by applying the following formula:

\[
\text{Titer (HCT116 viral genome /ml)} = \frac{\text{Number of target cells (count at day 1) } \times \text{ number of copies per cell of the sample}}{\text{Volume of supernatant in (mL)}}
\]

*Using standard DNA extraction procedures in a laboratory context where HIV sequences are often handled, one can expect a level of background contamination with HIV sequences corresponding to cells containing 1 copy per 1000 genomes. In this case, consider higher copy numbers for calibration. Vector stocks failing to give higher than 0.01 copy per genome in a QPCR assay have an infectivity index that renders them unusable for most applications (see Anticipated Results). Using careful DNA extraction procedures and standardization as described above, one can expect reproducibility within a 2-fold range. Investigators should consult a local QPCR expert if a more stringent quantitative PCR procedure is needed.*