

Takara Bio USA, Inc.

Lenti-X™ Provirus Quantitation Kit User Manual

Cat. No. 631239
(050819)

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I. Introduction

A. Summary

The Lenti-X Provirus Quantitation Kit uses qPCR and TB Green® chemistry to determine the number of integrated provirus copies in a mixed or clonal population of cells transduced with HIV-1-based lentivirus. To perform the analysis, genomic DNA (gDNA) is extracted from transduced cells using the provided Nucleospin® Tissue Kit. Serial dilutions of the cellular gDNA are then subjected to qPCR amplification alongside dilutions of a calibrated Provirus Control Template, which are used to generate a standard curve. Since the viral sequences in gDNA and the Control Template are amplified with different PCR sensitivities, a correction coefficient is incorporated in order to calculate the provirus copy number from the total qPCR copy number that was found using the standard curve. After determining the cell number equivalents represented in the gDNA yield using the generic conversion of 6.6 pg gDNA/genome, the final result is expressed in terms of provirus copies/cell. The method is universal to most HIV-1 vectors and can be used with vectors that do not carry fluorescent or antibiotic markers.

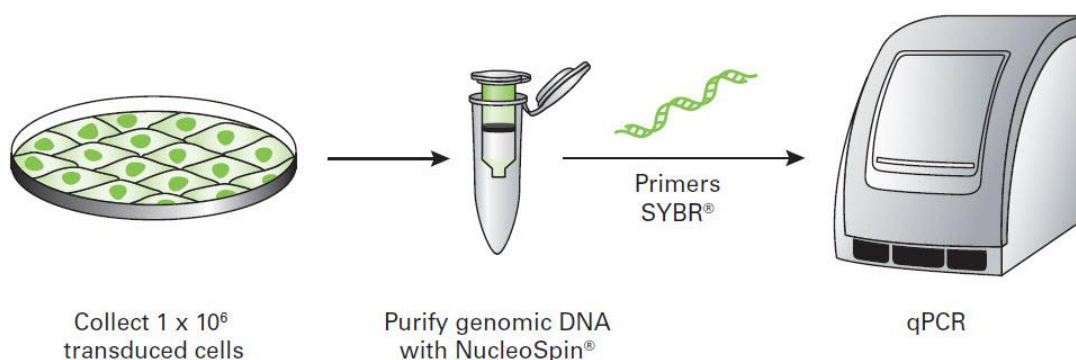


Figure 1. Flowchart of the procedure for determining provirus copy number using the Lenti-X Provirus Quantitation Kit

II. List of Components

Store the Lenti-X Provirus Quantitation Components at -20°C .

Store the TB Green Advantage® qPCR Premix and ROX Reference Dyes (Cat. No. 639676) at -70°C in the dark. After thawing, store at 4°C in the dark. Do not refreeze.

Store the NucleoSpin Tissue Kit at room temperature.

Box 1: Lenti-X Provirus Quantitation Components (Cat. No. 631240; not sold separately)

- 30 μl Lenti-X Provirus Control Template (5×10^5 copies/ μl)
- 100 μl Lenti-X Provirus Forward Primer (10 μM)
- 100 μl Lenti-X Provirus Reverse Primer (10 μM)
- 4 tubes EASY Dilution Buffer (1 ml/tube)

Box 2: NucleoSpin Tissue Kit (10 preps; Cat. No. 740952.10)

See the *Genomic DNA from Tissue User Manual* (PT4010-1) for a list of components and storage conditions.

Box 3: TB Green Advantage qPCR Premix (200 rxns; Cat. No. 639676)

- 4 tubes TB Green Advantage qPCR Premix (2X; 0.625 ml/tube)
- 100 µl ROX Reference Dye LSR (50X)
- 100 µl ROX Reference Dye LMP (50X)

User Manuals

User manuals for Takara Bio products are available for download at takarabio.com/manuals. The following user manuals apply to this product:

- Lenti-X Provirus Quantitation Kit User Manual (PT5112-1)
- TB Green Advantage qPCR Premix User Manual (PT3883-1)
- Genomic DNA from Tissue User Manual (PT4010-1)

III. Additional Materials Required

- PCR-qualified work areas and pipettors free of contaminating DNA and DNases.
- Quantitative real-time PCR thermal cycler (e.g., Mx3000P®, Stratagene; ABI 7900, Applied Biosystems; or equivalent)
- Ethanol (96–100%)
- PCR-grade water
- 96-well PCR plates and 8-well PCR strips
- Repeating pipettor with capacity of up to 23 µl (Section IV.D)
- Multichannel pipettor(s) with 2–25 µl capacity
- 1.5 ml microtubes for sample lysis and DNA elution

IV. Provirus Quantitation Protocols

PLEASE READ THESE PROTOCOLS IN THEIR ENTIRETY BEFORE STARTING

Successful results depend on performing the following steps properly and in sequence.

A. General qPCR Recommendations

Due to the tremendous amplification power and sensitivity of qPCR, even trace amounts of contaminating DNA will be amplified and will affect Ct and final copy number values. Before you begin, prepare work areas free of potentially contaminating DNA and DNases. If possible, dilute your samples and controls in one work area with a dedicated set of pipettors, and assemble your qPCR reactions in a separate area or noncirculating containment hood, using a different set of dedicated pipettors. Wear gloves at all times and use PCR pipette tips with hydrophobic filters, and dedicated solutions. We also recommend setting up negative template control (NTC) reactions lacking any template. Finally, perform all post-PCR analyses in a separate area, preferably in a separate room, with different pipettors.

B. Preparing Buffers for the NucleoSpin Tissue Kit

Before using the NucleoSpin Tissue Kit for the first time, be sure to read the Genomic DNA from Tissue User Manual (PT4010-1) and prepare the working buffer solutions as stated in the manual. For additional details, consult the user manual.

Caution: NucleoSpin Tissue Buffers B1, B3, and BW contain guanidine hydrochloride! Wear gloves and goggles!

C. Protocol: Purifying Genomic DNA from Transduced Cells—1 hr

1. At least 72 hrs after infecting your target cells with lentivirus, transfer $\sim 1 \times 10^6$ transduced cells into a micro-centrifuge tube and collect the cells by centrifugation. Discard supernatant.

NOTE: Due to the transient formation of lentiviral DNA episomes that arise soon after infection, transduced cells should not be analyzed until 72 hrs after infection.

2. Using the NucleoSpin Tissue Kit, follow the standard protocol in the Genomic DNA from Tissue User Manual (PT4010-1) to isolate genomic DNA from cultured cells.
3. Elute the DNA in a volume of 100 μ l prewarmed **Buffer BE** (-70°C). Measure the OD_{260} to determine DNA yield and concentration. The extraction should yield 20–30 μ g of DNA at a concentration of 200–300 ng/ μ l.
4. (Optional) You may check the quality of the purified DNA by running 1–2 μ l of the eluate on a 1.2% agarose gel. We generally see a band at 23 kb, which may be accompanied by some faint smearing (Figure 2).



Figure 2. Examples of genomic DNA purified using the NucleoSpin Tissue Kit. Lanes 1 & 2: purified genomic DNA. Lane 3: DNA markers.

D. Protocol: qPCR Amplification of Provirus Sequences in Genomic DNA—3 hr

1. Master Mix Preparation

In your PCR reaction assembly work area, and on ice, assemble a sufficient volume of Master Reaction Mix (MRM) specific for your qPCR instrument using the reagents listed in Table I. Add the TB Green Advantage qPCR Premix last and keep on ice. *To ensure that you will have enough MRM, prepare approximately 10% more than the minimum required for your experiment (Table 2).* Including all duplicates, each experiment requires 16 Control/NTC reactions and 8 reactions for each gDNA sample. All reactions should be performed in duplicate:

Table 1. Master reaction mixes recommended for different qPCR instruments

Reagent	qPCR Instrument			
	Stratagene MX3000P	Takara Bio Thermal Cycler Dice™ Real Time System	Applied Biosciences Instruments	Roche LightCycler
Regent volume (µl/well) for each instrument				
PCR-grade H ₂ O	9.0	9.5	6.8	7.2
Lenti-X Provirus Forward Primer (10 µM)	0.5	0.5	0.4	0.4
Lenti-X Provirus Reverse Primer (10 µM)	0.5	0.5	0.4	0.4
ROX Reference Dye LSR or LMP (50X)*	0.5	—	0.4	—
TB Green Advantage qPCR Premix (2X)	12.5	12.5	10.0	10.0
Total (µl/well)	23.0	23.0	18.0	18.0

* The kit is supplied with two different ROX formulations that allow you to normalize fluorescence signals on instruments equipped with this option. ROX Reference Dye LSR is for instruments whose excitation source is a 488 nm laser, while ROX Reference Dye LMP is for instruments whose excitation source is either a lamp or an LED. **Be certain to use the formulation that is appropriate for your real-time instrument!**

NOTE: To ensure sufficient pipetting volume, prepare approximately 10% more Master Reaction Mix than the minimum amount needed for the experiment. For convenience, use Table 2 below:

Table 2. Master reaction mix preparation chart (example for Mx3000P).

Reagent	Volume (µl/well)	Total wells	Total volume	Total + 10%
PCR-grade H ₂ O	9.0			
Lenti-X Provirus Forward Primer (10 µM)	0.5			
Lenti-X Provirus Reverse Primer (10 µM)	0.5			
ROX Reference Dye LSR or LMP (50X)*	0.5			
TB Green Advantage qPCR Premix (2X)	12.5			
Total (µl/well)	23.0			

*Be certain to use the formulation that is appropriate for your real-time instrument! See Table 1.

2. Sample and Control Dilutions

In your sample dilution work area, and using PCR grade 8-well strips, prepare dilutions of the Control Template and your purified gDNA sample(s) using EASY Dilution Buffer as shown in Table 3.

1. First, add the required amount of EASY Buffer to the wells of each strip, as shown in Table 3.
2. Controls: Dilute the Lenti-X Provirus Control Template in an 8-well strip (Table 3, 'Strip 1') as follows:
3. In Well #1 of Strip 1, add 2 µl of the Lenti-X Provirus Control Template stock into 18 µl of EASY Buffer for a 1:10 dilution (diluted sample = 5×10^4 copies/µl). Mix gently and thoroughly.
4. Make subsequent 1:5 dilutions of the control in Wells #2 – #6 by serially transferring 4 µl of the preceding dilution into the 16 µl of EASY Buffer in the next well. Mix gently and thoroughly before making the next dilution.

5. **NTCs:** The EASY Buffer in Wells #7 & #8 is used for the NTC controls. Be sure not to contaminate these wells with any DNA.
6. **Samples:** Dilute your purified gDNA sample(s) using one or more 8-well strips. Each strip can be used to make dilutions for 2 samples (Table 3, ‘Strip 2, etc.’).
7. In a clean microfuge tube, dilute a portion of each purified gDNA sample to 50 ng/μl using EASY buffer. Add 20 μl of the diluted gDNA to the first well in each dilution series (i.e. Wells #1 or #5).
8. Subsequent 5-fold sample dilutions (wells 2–4 & 6–8) can be made by serially transferring 4 μl of sample from one well into 16 μl of buffer in the next well.
9. Centrifuge the strips at 2000 rpm (4°C) for 1 min to remove any bubbles.

Table 3. Control template and sample dilutions for qPCR^a

Well #	Strip 1: Controls/Standard Curve			Strip 2: gDNA Samples 1 & 2		
	EASY Buffer	Control Template	Copies/qPCR rxn	EASY Buffer	Control Template	Copies/qPCR rxn
1	18 μl	2 μl	1 x 10 ⁵	—	20 μl ^b	100
2	16 μl	4 μl of #1	2 x 10 ⁴	16 μl	4 μl of #1	20
3	16 μl	4 μl of #2	4 x 10 ³	16 μl	4 μl of #2	4
4	16 μl	4 μl of #3	8 x 10 ²	16 μl	4 μl of #3	0.8
5	16 μl	4 μl of #4	1.6 x 10 ²	—	20 μl ^c	100
6	16 μl	4 μl of #5	3.2 x 10 ¹	16 μl	4 μl of #5	20
7	16 μl	—	0 (NTC)	16 μl	4 μl of #6	4
8	16 μl	—	0 (NTC)	16 μl	4 μl of #7	0.8

^a 2 μl of each dilution are used in each respective qPCR reaction

^b Sample 1, 50 ng/μl

^c Sample 2, 50 ng/μl

3. qPCR Reaction Assembly

In your PCR reaction assembly area, place a 96-well PCR plate on ice (or on a blueblock; 4°C), and dispense the appropriate total volume of MRM/well for your thermal cycler into the appropriate wells (in duplicate) using a repeating pipettor (e.g., 23 μl/well for Stratagene’s Mx3000P, see Table 1).

1. Using a multichannel pipettor, transfer 2 μl/well of the control dilutions, NTCs, and sample dilutions (in duplicate) from the 8-well PCR strips to the PCR plate containing MRM.
2. We recommend that you program your real-time qPCR instrument for the following qPCR reaction cycles (see Table 4). Include a final dissociation curve cycle.

Table 4. Recommended thermal cycling conditions for different qPCR instruments.

Reaction cycles	qPCR Instrument									
	Stratagene MX3000P		Takara Bio Thermal Cycler Dice Real Time System		ABI7500 fast		ABI7000		Roche LightCycler	
	Thermal cycling conditions for each instrument									
Initial denaturation (1 cycle):	95°C	10 sec	95°C	30 sec	95°C	30 sec	95°C	30 sec	95°C	30 sec
qPCR (40 cycles):	95°C	5 sec	95°C	5 sec	95°C	5 sec	95°C	5 sec	95°C	5 sec
	60°C	20 sec	60°C	30 sec	60°C	25 sec	60°C	31 sec	60°C	20 sec
Dissociation curve (1 cycle):	95°C	1 min	95°C	15 sec	95°C	15 sec	95°C	15 sec	95°C	0 sec ^a
	60–95°C	30 sec	60°C	30 sec	60°C	1 min	60°C	1 min	60°C	15 sec
			95°C	15 sec	95°C	15 sec	95°C	15 sec	95°C	0 sec ^b

^a 20°C/sec

^b 0.1°C/sec

NOTE: Although Table 4 shows optimized cycling conditions for a selection of commonly used instruments, the Lenti-X Provirus Quantitation Kit can be used with a variety of real-time instruments and is not limited to those listed in the table. For instruments not listed, please refer to our TB Green qPCR Premix User Manual (PT3883-1) and/or your instrument's user manual to determine cycling conditions for your particular thermal cycler.

4. Data Analysis and Calculations

Briefly, the Ct values from your gDNA sample amplification plots are used to determine the qPCR copy number equivalents from the standard curve. Total qPCR copy number equivalents are then determined for each original sample, which are then corrected for the difference in qPCR sensitivity of amplifying plasmid vs. gDNA amplimers to determine total provirus copy number. The total number of genomes for each sample is calculated from the gDNA yield using an accepted constant of 6.6 pg DNA/cell, and these two values are used to finally determine the provirus copy number/cell (or genome).

1. Generate a standard curve.

Determine average Ct values for each pair of duplicate control template amplifications and plot the average Ct vs. copy number on a log scale to generate a standard curve (Figure 3B). Important: the plotted qPCR standard curve should have a PCR efficiency of 0.9–1.1 and an $R^2 > 0.990$.

2. Determine the qPCR copy number equivalent.

Determine average Ct values for each pair of duplicate sample amplifications and use each average Ct value to read the corresponding qPCR copy number equivalent from the standard curve. Use sample Ct values that are below that of the NTC, but do not use any sample Ct values that are below that of the 32-copy standard (i.e., off the curve).

3. Determine total qPCR copy number equivalent for original sample.

- Determine the overall dilution factor for each 100, 20, 4, or 0.8 ng sample reaction that yielded a qualified qPCR copy number equivalent by using the following equation:

$$\text{DNA dilution factor} = ([\text{Total DNA extracted}] \mu\text{g})(1000 \text{ ng}/\mu\text{g}) / ([100, 20, 4, \text{ or } 0.8] \text{ ng})$$

- Multiply the qPCR copy number equivalent by its corresponding dilution factor to compute the total plasmid qPCR copy number equivalent for each sample dilution.
 - Use the total values computed for each sample reaction in Step 3b to calculate the mean total qPCR copy number equivalent for the original gDNA sample.
- ##### 4. Convert total qPCR copy number equivalent to provirus copy number.

Quantitative PCR sensitivity is context-dependent and detects amplimers in gDNA much less efficiently than it detects them in plasmid DNA. As a result, the qPCR copy number underestimates the actual provirus copy number present in the genome. It is therefore necessary to convert the total qPCR copy numbers to provirus copy numbers by using a correction factor of 62.84 provirus copies/qPCR copy* as follows:

$$\text{Provirus copy number} = (\text{qPCR copy number})(62.84 \text{ provirus copies}/\text{qPCR copy})$$

*This correction factor was determined empirically at Takara Bio USA, Inc.

5. Calculate the total genome number equivalents present in the original gDNA sample.

Use the generic conversion of 6.6 pg gDNA/cell to calculate the total number of genomes (i.e., cells) represented in your purified gDNA sample(s).

$$\text{Cell number} = ([\text{gDNA}] \mu\text{g})(1 \times 10^6 \text{ pg}/\mu\text{g})/(6.6 \text{ pg}/\text{cell})$$

6. Calculate provirus copy number/cell.

Finally, divide the total provirus copy number (from Step 4) by the cell number (from Step 5) to yield provirus copy number/cell.

$$\text{Provirus copy number/cell} = (\text{provirus copy number})/(\text{cell number})$$

NOTE: The 6.6 pg gDNA/cell constant is calculated based on the molecular weight of nuclear DNA in a normal, diploid human cell. An alternative method of normalizing your result for the number of cells or genomes represented in the gDNA sample for your particular cell line, is to determine (in parallel) the copy number of a cellular or housekeeping gene (e.g., albumin). This requires the use of a second plasmid standard which contains the control gene in order to generate a second standard curve (Lizée et al 2003 and Sastry et al 2002).

V. Provirus Quantitation Examples and Calculations

The following examples of standard curve and sample data, and the accompanying calculations, are provided to illustrate the determination of provirus copy number/cell for a typical sample of purified genomic DNA.

- The raw qPCR amplification plots for the dilutions of the Provirus Control Template are shown in Figure 3A. The Ct values from these plots (for a threshold of 10^{-1} dRn) are listed in Table V and were used to construct the standard curve shown in Figure 3B. Note that the PCR efficiency and R^2 values are within the required range.

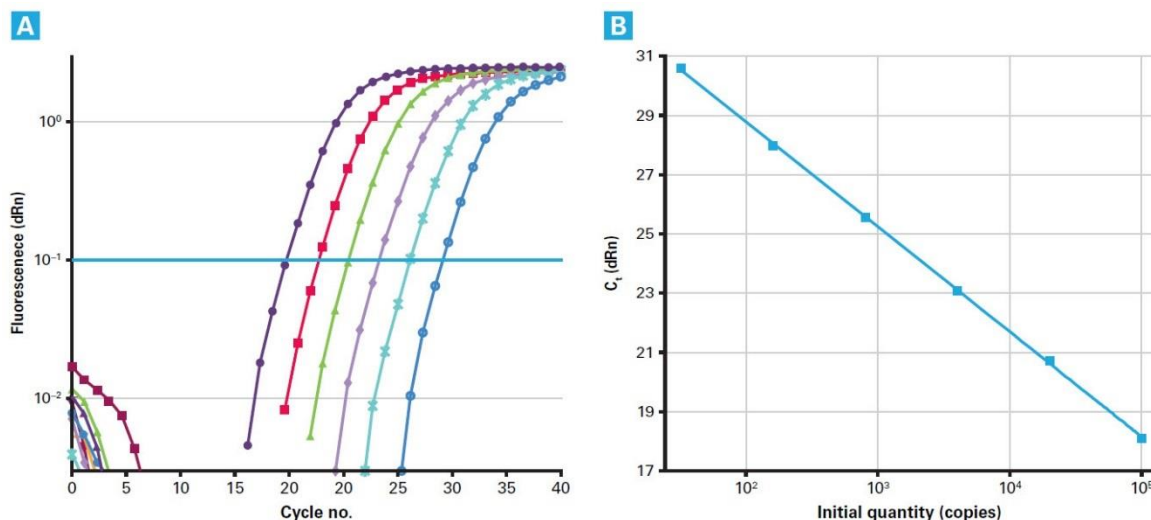


Figure 3. Using the Lenti-X Provirus Control Template to generate a standard curve. Panel A. qPCR amplification plots of serial dilutions of the Lenti-X Provirus Control Template (10^5 –32 copies). **Panel B.** A standard curve created from the plots shown in Panel A demonstrates a strong linear correlation between the Ct values of the serial dilutions and copy number (log scale), with $R^2 = 1.00$ and a PCR efficiency of 91.6%.

Table 5. Example standard curve raw data.

Well	Copies	Ct	Tm of product
Standard 1	1 x 10 ⁵	18.13	78.8
Standard 2	2 x 10 ⁴	20.71	78.7
Standard 3	4000	23.06	78.8
Standard 4	800	25.53	78.6
Standard 5	160	27.97	78.7
Standard 6	32	30.59	78.6
NTC	0	None	61.8
NTC	0	None	62.9
Threshold (dRn)	R ² (dRn)	Slope (dRn)	Efficiency (%)
0.1	1	-3.54	91.6

- The Ct values from the amplification plots of the gDNA sample dilutions (not shown) are then found on the standard curve to determine the corresponding qPCR copy number for each dilution (Table 6). Each of these raw qPCR copy number values is then corrected by its specific dilution factor to compute the total qPCR copy numbers represented in the original sample. These total values are averaged to yield a mean total copy number value.

Table 6. Example sample raw data

gDNA	Ct	qPCR copies ^a	Dilution factor ^b	Total qPCR copies in sample	Mean total copies
100 ng	22.36	6060	300	1.81 x 10 ⁶	2.07 x 10 ⁶
20 ng	24.29	1550	1,500	2.33 x 10 ⁶	
4 ng	26.61	302	7,500	2.36 x 10 ⁶	
0.8 ng	29.15	50.2	3.75 x 10 ⁴	1.88 x 10 ⁶	

a As read from the standard curve (Figure 2B).

b Based on a gDNA yield of 30 µg

c Total qPCR copies = (qPCR copies)(Dilution factor)

- The total qPCR copy number for the sample is then used to find the provirus copy number in the gDNA using the equation in Section E, Step 4.

$$\text{Provirus copy number} = (2.07 \times 10^6 \text{ qPCR copies})(62.84 \text{ provirus copies/qPCR copy}) = 1.30 \times 10^8 \text{ copies}$$

- The number of cells (i.e., genomes) represented in the purified gDNA sample is calculated using the equation shown in Section E, Step 5.

$$\text{Cell number} = (30 \mu\text{g gDNA})(1 \times 10^6 \text{ pg}/\mu\text{g})/(6.6 \text{ pg gDNA}/\text{cell}) = 4.55 \times 10^6 \text{ cells}$$

- Finally, the provirus copy number per cell is determined by dividing the provirus copy number by the cell number. Final results are listed in Table VII.

$$\text{Provirus copy number}/\text{cell} = (1.30 \times 10^8 \text{ copies})/(4.55 \times 10^6 \text{ cells}) = 28.59 \text{ provirus copies}/\text{cell}$$

Table 7. Example final results.

Total gDNA	qPCR copies	Total provirus copies ^b	Total cellular genomes ^a	Provirus copies/cell
30 µg	2.07×10^6	1.30×10^8	4.55×10^6	28.59

a Total cellular genomes present in gDNA sample (6.6 pg gDNA/cell genome)

b Total provirus copies = (Total qPCR copies)(62.84 provirus copies/qPCR copy)

VI. References

Lizée, G., *et al.* Real-Time Quantitative Reverse Transcriptase-Polymerase Chain Reaction as a Method for Determining Lentiviral Vector Titers and Measuring Transgene Expression. *Hum. Gene Ther.* **14**:497–507 (2003).

Sastry, L., *et al.* Titering lentiviral vectors: comparison of DNA, RNA and marker expression methods. *Gene Ther.* **9**:1155–1162 (2002).

Appendix A. Troubleshooting Guide

Table 8. Troubleshooting guide for Lenti-X Provirus quantitation

Problem	Possible Explanation	Solution
High signal in NTC reactions	Contamination of buffer, pipets, or work area from improper handling of samples or control template	Diagnosis: Run control and NTC qPCR samples on 3% agarose gel to visually compare size of product bands (virus-specific vs. nonspecific) and compare dissociation curves. The size of the specific amplicon used for quantitation is ~140 bp. Dissociation curves should reflect the presence of a single product of this size, which should also be visible in the agarose gel.
		Prepare work area properly and use clean, dedicated pipets for each phase of the protocol: dilution, reaction set-up, and analysis.
Poor efficiency or R²	Poor technique or inconsistent pipetting	Review qPCR techniques; use repeating pipettors and multichannel pipettors for improved accuracy; calibrate pipets.
Provirus gDNA amplification signal is higher than expected	High proviral copy number or excess gDNA was added to reactions	Check gDNA concentration and redilute if necessary. Be sure that only 100 ng of gDNA was added to the first well of the qPCR.
Provirus gDNA amplification-signal is absent or lower than expected	Insufficient gDNA used in reactions	Check gDNA concentration and redilute
	Low provirus copy number due to low virus titer	Concentrate the virus in your supernatant and repeat transduction. We recommend using Lenti-X Concentrator (Cat. No. 631231)
	Low provirus copy number due to poor transduction efficiency	<ul style="list-style-type: none"> Infect your target cells using higher MOI(s). Select or sort the cell population to isolate clones or enrich for transduced cells. If possible, use spinoculation or add infection enhancers (i.e., RetroNectin® [Cat. No. TAK T100A] or polybrene) to improve infection kinetics and result in higher effective MOIs. Some cell lines are more susceptible than others to VSV-G lentiviral infection. Validate the infectivity of your specific cell line by using a lentivirus that contains a marker gene (e.g. ZsGreen1 or LacZ) and assess transduction efficiency by another means prior to assaying for provirus. For more information regarding effective lenti-viral transduction methods, consult our Lenti-X Lentiviral Expression Systems User Manual (PT3883-1; see Sections VII & VIII, and Appendix B).

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This document has been reviewed and approved by the Quality Department.