# Chapter 12

# Transient Expression of Green Fluorescent Protein in Integrase-Defective Lentiviral Vector-Transduced 293T Cell Line

**Fazlina Nordin, Zariyantey Abdul Hamid, Lucas Chan, Farzin Farzaneh, and M.K. Azaham A. Hamid**

## Abstract

Non-integrating lentiviral vectors or also known as integrase-defective lentiviral (IDLV) hold a great promise for gene therapy application. They retain high transduction efficiency for efficient gene transfer in various cell types both in vitro and in vivo. IDLV is produced via a combined mutations introduced on the HIV-based lentiviral to disable their integration potency. Therefore, IDLV is considered safer than the wild-type integrase-proficient lentiviral vector as they could avoid the potential insertional mutagenesis associated with the nonspecific integration of transgene into target cell genome afforded by the wild-type vectors.

Here we describe the system of IDLV which is produced through mutation in the integrase enzymes at the position of D64 located within the catalytic core domain. The efficiency of the IDLV in expressing the enhanced green fluorescent protein (GFP) reporter gene in transduced human monocyte (U937) cell lines was investigated. Expression of the transgene was driven by the spleen focus-forming virus (SFFV) LTRs. Transduction efficiency was studied using both the IDLV (ID-SFFV-GFP) and their wild-type counterparts (integrase-proficient SFFV-GFP). GFP expression was analyzed by fluorescence microscope and FACS analysis.

Based on the results, the number of the GFP-positive cells in ID-SFFV-GFP-transduced U937 cells decreased rapidly over time. The percentage of GFP-positive cells decreased from ~50% to almost 0, up to 10 days post-transduction. In wild-type SFFV-GFP-transduced cells, GFP expression is remained consistently at about 100%. These data confirmed that the transgene expression in the ID-SFFV-GFP-transduced cells is transient in dividing cells. The lack of an origin of replication due to mutation of integrase enzymes in the ID-SFFV-GFP virus vector has caused the progressive loss of the GFP expression in dividing cells.

Integrase-defective lentivirus will be a suitable choice for safer clinical applications. It preserves the advantages of the wild-type lentiviral vectors but with the benefit of transgene expression without stable integration into host genome, therefore reducing the potential risk of insertional mutagenesis.

**Key words** Integrase-defective lentiviral vector, D64 point mutation, Transduction efficiency, GFP reporter gene, U937 cell lines

Maurizio Federico (ed.), *Lentiviral Vectors and Exosomes as Gene and Protein Delivery Tools*, Methods in Molecular Biology, vol. 1448, DOI 10.1007/978-1-4939-3753-0_12, © Springer Science+Business Media New York 2016

## 1 Introduction

Viral vector systems based on lentiviruses have been extensively analyzed and used for gene therapy applications [1–3] due to their several advantageous properties [4, 5] over other viral vectors. Moreover, lentiviral vectors have been shown to be capable in transducing both dividing and nondividing cells, including stem cells which allow higher level of gene delivery to these cells [6–8].

Despite all of the advantages concerning the use of lentiviral vectors, a number of problems [9] have limited their use. These include the risk of insertional mutagenesis and subsequent malignant transformation of the transduced cells which is afforded by their stable integration to the genome of host cells [10, 11]. Thus, the integrase-defective lentiviral vectors (IDLVs) have been developed to overcome these limitations. IDLVs have been shown to mediate efficient gene expression both in vitro and in vivo with a lower risk of insertional mutagenesis, and thus offer an invaluable prospect in the field of gene therapy [5, 12–14].

IDLVs can be produced through combined mutations into enzyme integrase domains which are consisted of three functional protein domains as follows: (1) N-terminal domain, (2) the catalytic core domain, and (3) the C-terminal domain [15, 16]. The mutations were made to disable the viral RNA integration in the host genome while maintaining the transgene expression episomally in order to minimize the risk of insertional mutagenesis [17].

In this study, we describe the system of IDLV produced through D64 amino acid point mutations located within the catalytic core domain. This type of mutation is commonly used to establish IDLV [18, 19]. ID-SFFV-GFP and their wild-type counterparts (SFFV-GFP) were produced and transduced into U937 cells at multiplicity of infection (MOI) 5, and GFP expression was analyzed using fluorescence microscope and FACS analysis.

## 2 Materials

Prepare all solutions using deionized water or ultrapure water (prepared by purifying deionized water to attain a sensitivity of 18 MΩ cm at 25 °C) and analytical grade reagents. Prepare and store all reagents at room temperature unless indicated otherwise. Follow all safety regulations including waste disposal procedure when disposing waste materials. Pay close attention to hazardous materials and follow the SOP as provided in the laboratory.

### 2.1 Lysis Buffer (LyB)

10 mL LyB stock: 20 mM HEPES, 50 mM sodium chloride ($NaCl_2$), 10 mM sodium fluoride (NaF), 1 mM ethylene glycol tetraacetic acid (EGTA), 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 2.0% Nonidet P40, 0.5% sodium

deoxycholate, 0.5% sodium dodecyl sulfate (SDS), and 10 µL protease inhibitor cocktail (*see* **Note 1**). Mix and add water. Aliquot into 1.5 mL tube. Store at –20 °C.

### 2.2 2× Laemmli Sample Buffer with β-Mercaptoethanol

10 mL of Laemmli Sample Buffer (LSB) stock: 100 mM Tris–HCl (pH 6.8), 2% SDS, 20% glycerol, and 4% β-mercaptoethanol (added fresh). Mix well and make up to 10 mL water. Aliquot into 1.5 mL tube. Store at –20 °C.

### 2.3 Tris-Buffered Saline

10× of Tris-buffered saline (TBS) stock buffer: 1 M Trizma base, and 1.5 M $NaCl_2$. Mix and make up to 1 L water. Dilute 10× TBS with water at 1:9 to make 1× working solution. Store at room temperature.

### 2.4 Blocking Buffer (Bb)

Bb stock buffer: 1× TBS, 7.5 g skimmed milk powder (*see* **Note 2**), and 0.1% Tween-20. Mix and make up to 150 mL water. Store at 4 °C, and use within 1 month from the date of preparation.

### 2.5 Antibody Buffer (Ab)

Ab stock buffer: 1× TBS, 1.0 g bovine serum albumin (faction V), and 0.01% sodium azide (*see* **Note 3**). Mix and make up to 20 mL water. Store at 4 °C, and use within 1 month from the date of preparation.

### 2.6 2× HeBSS

2× HeBSS stock buffer: 50 mM BES (*N*,*N*-bis[2-hydroxyethyl]-2aminuteoethanesulfonic acid), 280 mM NaCl, and 1.5 mM $Na_2HPO_4$. Mix and adjust pH with HCL to 6.96 (*see* **Note 4**). Make up to 1 L water. Store at room temperature.

### 2.7 Resolving Gel Buffer 10%

Mix all of these chemicals and reagents: 3 mL 30% Bis-acrylamide, 2.5 mL 1.5 M Tris–HCl (pH 8.8), 100 µL 10% APS, 100 µL 10% SDS, and 4 µL TEMED. Add 4 mL of water. Pour the mixture slowly in the disposable cassette (*see* **Note 5**). Let it stand for 30 min or until the gel is solid.

### 2.8 Stacking Gel Buffer 5%

Mix all of these chemicals and reagents: 0.67 mL 30% Bis-acrylamide, 0.5 mL 1.5 M Tris–HCl (pH 6.8), 40 µL 10% APS, 40 µL 10% SDS, and 4 µL TEMED. Add 2.7 mL of water. Layer the mixture slowly onto the solid resolving gel in the disposable cassette (*see* **Note 6**). Carefully insert and fix the combs into the cassette. Let it stand for 30 min or until the gel is solid. Keep the pre-cast gel in the container with 1× running buffer (Rb) covering the whole cassette (*see* **Note 7**). Store at 4 °C.

### 2.9 Antibodies and Conjugates

1. Primary antibody: Rabbit polyclonal GFP antibody (Cell Signaling), and mouse monoclonal α-tubulin antibody (Sigma).
2. Secondary antibody: Goat anti-rabbit HRP antibody, and goat anti-mouse HRP antibody. Both are from Santa Cruz Biotech. Inc.

### 2.10 Commercial Kits

1. ECL plus detection kit (GE Healthcare Life Sciences) (*see* **Note 8**).
2. Lenti-X™ qRT-PCR Titration Kit; Clontech, USA.
3. RNA isolation kit (Macherey-Nagel).

### 2.11 Cell Lines

Human embryo kidney (HEK) 293T cell lines are adherent cells, and will be used as packaging cells to produce lentivirus. Human monocyte cell lines (U937) are suspension cells and will be used as target cells to determine virus titre.

### 2.12 Culture Media

293T cell lines: Dulbeccos's modified Eagle medium (DMEM) (Sigma Aldrich) supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS) (PAA, Laboratories), 100 μg/mL penicillin-streptomycin (Sigma Aldrich). U937 cell lines: RPMI 1640 (Roswell Park Memorial Institute) (Sigma Aldrich) supplemented with 10% heat-inactivated FCS, 100 μg/mL penicillin-streptomycin.

## 3 Methods

### 3.1 Vector Expression Plasmids

Briefly, clone the GFP cDNA into Rous Sarcoma lentivirus (RSV) vector backbone which contains two promoters: RSV promoter located upstream of the HIV-1 Rev Response Element (RRE) site, and the spleen-focus-forming virus (SFFV) promoter located downstream of the RRE site (Fig. 1). SFFV promoter, which is located at 5′LTR, drives the expression of the transgene. The lentiviral vector is produced by using combination of four helper plasmids (the four-plasmid system): MDG (envelope plasmid), Rev (packaging plasmid), integrase-defective or MDLg/pRR (packaging plasmid), and vector plasmid type (transfer plasmid). Hereafter the lentiviral vectors are referred to as SFFV-GFP (wild type), and ID-SFFV-GFP (integrase defective). Preparing of the vector expression plasmids is according to the standard molecular biology methods for cloning (*see* **Note 9**).

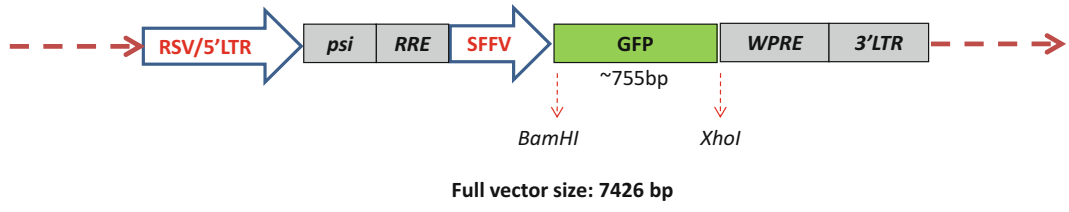


**Fig. 1** Schematic diagram of vector plasmid used as transfer plasmid to express transgene (GFP). GFP cDNA was cloned into *BamHI* and *XhoI* sites of RSV expression vector in-frame between SFFV (spleen focus-forming virus) promoter (5′LTR) and WPRE (3′LTR)

### 3.2 Calcium Phosphate ($Ca\text{-}PO_4$) Coprecipitation

Briefly, lentivirus is produced using the 293T cell line by seeding 4 million cells in 80 mL of complete media (DMEM supplemented with 10% heat-inactivated FBS (v/v), and 100 μg/mL penicillin-streptomycin) in 435 $cm^2$ triple-layer culture flask (*see* **Note 10**). The transfection of plasmids is performed using the standard calcium phosphate ($Ca\text{-}PO_4$) coprecipitation transfection protocol as described elsewhere with slight modification [20].

1. Perform the transfection according to the following recipes: 24 μg MDG plasmid, 40 μg MDLg/pRRE plasmid (either the wild type, or the integrase defective type), 20 μg Rev plasmid, and 80 μg vector plasmid.
2. Mix all plasmids in $dH_2O$ to final volume of 1977.3 μL. Add the same volume (1:1) of $CaCl_2$ (0.5 M) to the plasmid mixtures to make the final concentration of 0.25 M. Vortex the mixture.
3. Add the mixtures dropwise slowly (one drop every other second) to 1:1 of 2× HeBSS (pH 6.7) while vortexing at moderate speed (*see* **Note 11**). Incubate the DNA $Ca\text{-}PO_4$ coprecipitation mixture at room temperature for 30 min to form a fine opalescent precipitation (*see* **Note 12**).
4. Add the DNA $Ca\text{-}PO_4$ coprecipitation mixture into 80 mL of complete fresh media. Gently mix the mixture evenly. Pour the mixture slowly onto the inside of the triple-layer flask's bottle neck (*see* **Note 13**).
5. Secure the cap and let the flask stand for a few minutes, or when the mixture has settled evenly within the three compartments of the flask (*see* **Note 14**). Quickly lay the flask horizontally (*see* **Note 15**). Incubate at 37 °C with 5% $CO_2$. Replenish fresh complete media within 5–7 h post-transfection (*see* **Note 16**).
6. Harvest the culture medium containing the lentiviral vector particles at 48 h (*see* **Note 17**), and 72 h post-transfection by centrifugation at 300 × *g* for 10 min at 4 °C following filtration through 0.45 μM syringe filter. Mix both harvested lentiviral vector particles into one 250 mL centrifuge container.

### 3.3 Concentration of Lentiviral Vectors

1. Centrifuge the filtered culture medium containing the lentiviral vectors at 3900 × *g* overnight at 4 °C.
2. Discard the supernatant. Remove as much as supernatant from the pellet (*see* **Note 18**).
3. Dissolve pellet (which contains the lentiviral vectors) in 1 mL of serum-free media (X-Vivo 15) and aliquot in 0.2 mL tubes (*see* **Note 19**).
4. Keep the lentiviral vector at −80 °C and determine the titer by flow cytometry analysis or qRT-PCR (*see* **Note 20**).

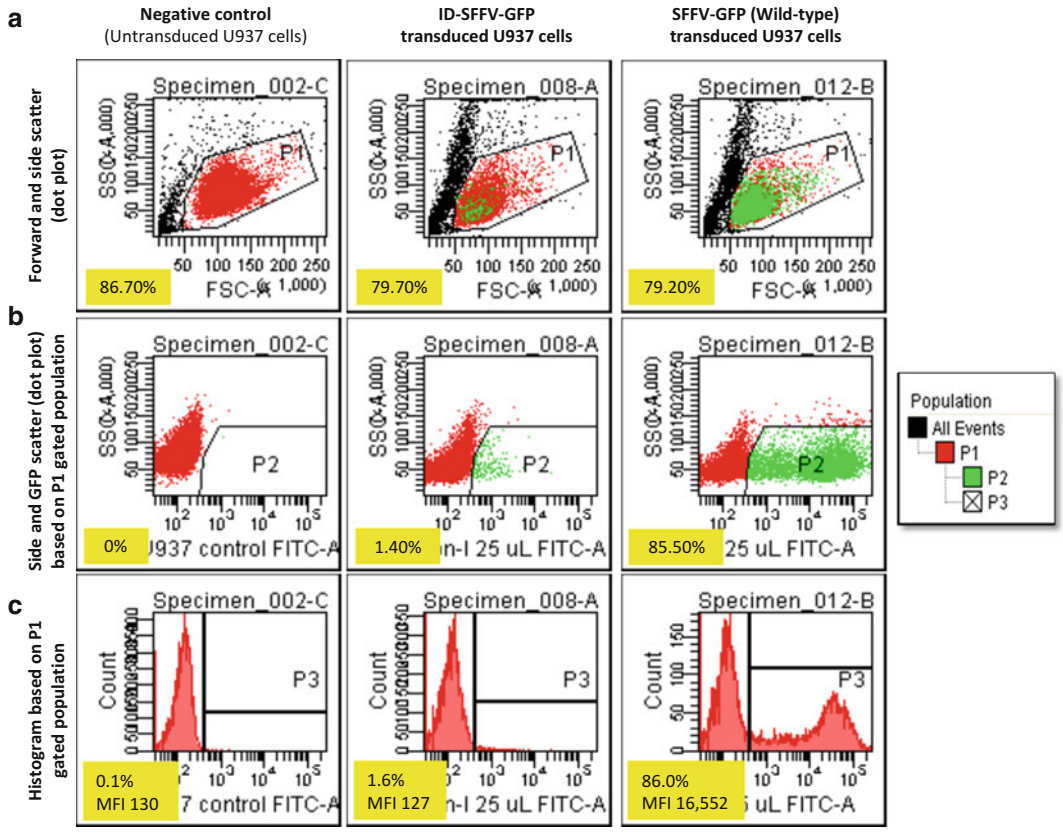


**Fig. 2** FACS analysis of ID-SFFV-GFP- and SFFV-GFP-transduced U937 cells on day 3 post-transduction (0.5 μL virus). (**a**) P1-gated region in the dot plot indicates the viable U937 population. (**b**) GFP (FITC-A)-specific side scatter was plotted based on P1-gated population to distinguish the GFP. (**c**) Histogram based on P1-gated population. GFP-positive cells were shifted to the right as labeled in P3 region of the histogram. Percentage and MFI of GFP-positive cells were taken from histogram as displayed in row "**c**." Non-transduced U937 cells were used as a negative control. *MFI* mean fluorescence intensity

### *3.4 Determination of Lentiviral Vector Titer by Flow Cytometry Analysis*

1. Perform this assay in triplicate for statistical analysis.
2. Transduce 200,000 U937 cells per mL with different volumes of concentrated virus in 10 μg/mL polybrene added prior to infection (*see* **Note 21**).
3. Incubate at 37 °C with 5% $CO_2$.
4. Determine titer at day 3 post-transduction in culture with 5–20% GFP-expressing cells (Fig. 2) (*see* **Note 22**).
5. Calculate lentiviral vector titer using this formula:

$$\frac{\text{Mean of GFP} - \text{expressing cells at day 3 post} - \text{transduction}\,(\%) \times \text{Total number of seeded cells} \times 1000\,\mu\text{L}}{* \ \mu\text{L of virus / vector}}$$

### 3.5 Qualitative Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR)

1. Use the RNA isolation kit to extract the lentiviral's RNA.
2. Elute RNA pellet in RNase-free $H_2O$, to the final concentration ranged between 55 and 90 μg/mL, and keep at −80 °C.
3. Use Lenti-X™ qRT-PCR Titration Kit to perform qRT-PCR.
4. Treat RNA with DNase (containing 1× DNase I buffer, 20 U DNase I enzyme, and RNase-free $H_2O$).
5. Incubate the mixture at 37 °C for 30 min, and then at 70 °C for 5 min.
6. Keep samples on ice for qRT-PCR analysis.
7. Perform qRT-PCR amplification in duplicate by mixing the viral RNA with master reaction mix (MRM) (Table 1).
8. Prepare Lenti-X RNA control template dilutions to generate a standard curve for determination of viral RNA copy numbers.
9. Perform samples analysis in a qPCR instrument (G-Storm, Gene Technologies Ltd., UK) (*see* **Note 23**) using the recommended qRT-PCR reaction cycles (Table 2).

**Table 1**
**Master reaction mix (MRM) for qRT-PCR**

| Regents | Volume/well (μL) |
|---|---|
| RNase-free water | 8.0 |
| Quant-X buffer (2×) | 12.5 |
| Lenti-X Forward primer (10 μM) | 0.5 |
| Lenti-X Reverse primer (10 μM) | 0.5 |
| ROX™ reference dye LSR | 0.5 |
| Quant-X enzyme | 0.5 |
| RT enzyme mix | 0.5 |
| Total | 23.0 |

**Table 2**
**qRT-PCR reaction cycles**

| Programs | Temperatures/duration |
|---|---|
| RT reaction | 42 °C/5 min<br>95 °C/10 s |
| qPCR×40 cycles | 95 °C/5 s<br>60 °C/30 s |
| Dissociation curve | 95 °C/15 s<br>60 °C/30 s<br>All (60–95 °C) |

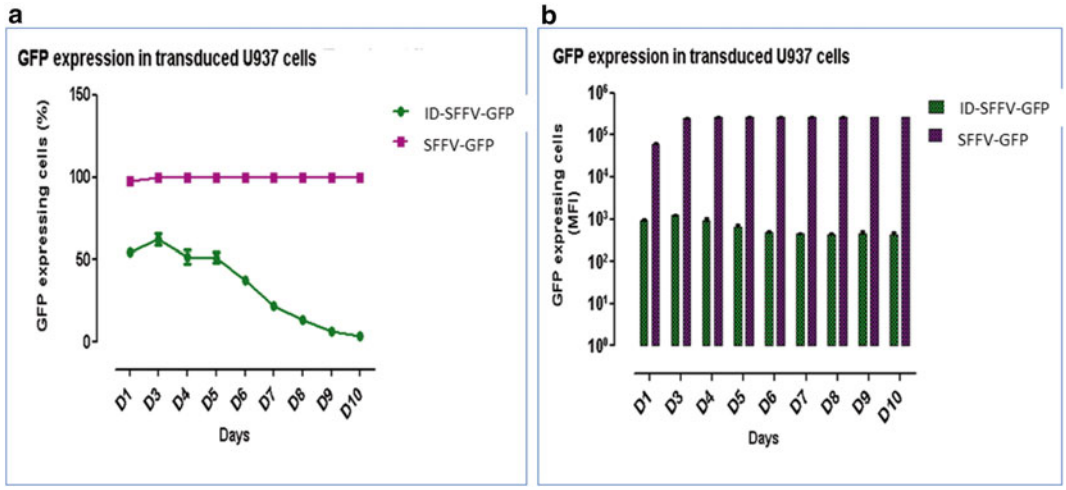


**Fig. 3** GFP expression in ID-SFFV-GFP- and SFFV-GFP-transduced U937 cells. A total of $5 \times 10^5$ U937 cells/2 mL were transduced with the indicated vectors (MOI 5) and further incubated up to 10 days. GFP expression was determined by FACS. (**a**) Percentage of GFP-expressing cells in SFFV-GFP- and ID-SFFV-GFP-transduced cells. The ID-SFFV-GFP -transduced U937 cells showed reduced GFP expression while GFP expression was consistently higher in the SFFV-GFP-transduced U937 cells. (**b**) Mean fluorescence intensity (MFI) in the SFFV-GFP- and ID-SFFV-GFP-transduced cells. Data shown are the mean ± standard deviation (SD) of triplicate samples

### *3.6 Transduction of U937 with Lentiviral Vectors*

1. Seed U937 cells at 500,000 cells per 2 mL in six-well plates and incubate overnight.
2. Day 0: Add 10 μg/mL of polybrene to each culture to increase transduction efficiency (*see* **Note 24**). Add either SFFV-GFP lentiviral (wild type) or ID-SFFV-GFP lentiviral at MOI 5 and further incubate for fluorescence microscopy, flow cytometry, and western blot analyses.
3. Days 1–10: Take 100 μL of U937-transduced lentiviral to determine the percentage of cell expressing GFP by flow cytometry analysis (Fig. 3).
4. Day 3: Make morphology observation of GFP expression using fluorescence microscopy (Fig. 4).
5. Day 3: Take 1 mL of U937-transduced lentiviral to detect GFP protein by western blot analysis (Fig. 5).

### *3.7 Preparation of Cell Lysates*

1. Wash twice $3 \times 10^6$ of cell pellet in DPBS.
2. Dissolve the cell pellet in 100 μL of LyB.
3. Incubate sample for 10 min on ice, and centrifuge for 10 min at 10,000 × *g* at 4 °C (*see* **Note 25**).
4. Store cell lysates at −20 °C.

### *3.8 SDS-PAGE Gel Electrophoresis*

1. Perform the gel electrophoresis using 10% SDS-polyacrylamide gradient gels (SDS-PAGE).
2. Mix protein with 2× LSB to 1:1.

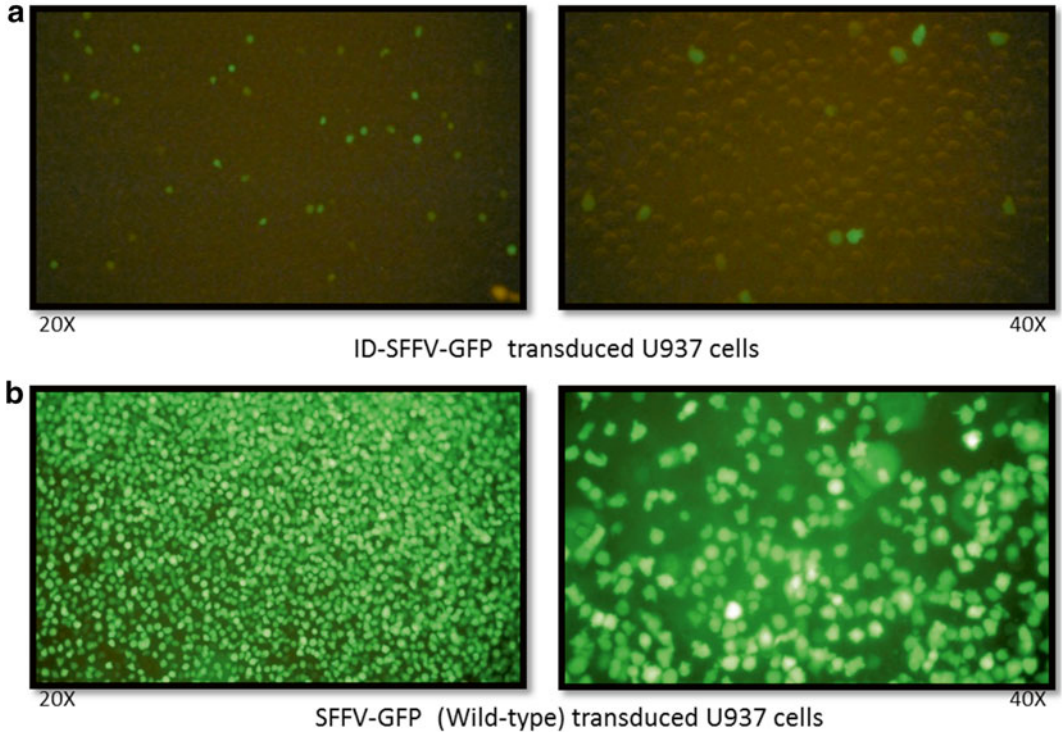


**Fig. 4** GFP expression in ID-SFFV-GFP- and SFFV-GFP-transduced U937 cells on day 3 post-transduction (MOI 5). (**a**) <5 % of ID-SFFV-GFP-transduced U937 cells showed green fluorescence indicating GFP expression. (**b**) Virtually all SFFV-GFP-transduced U937 cells showed GFP expression. The cells were viewed at 20× and 40× magnifications

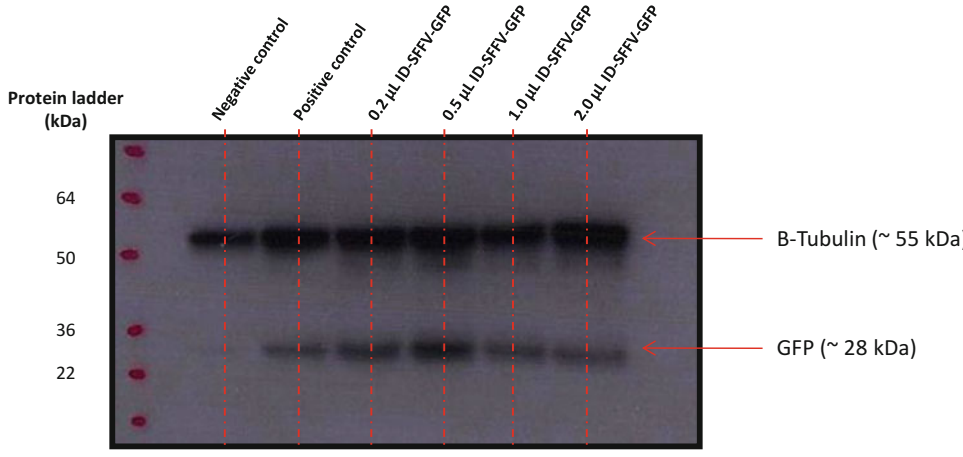


**Fig. 5** Western blot analysis of ID SFFV-GFF-transduced U937 cells. Intracellular expression (~35 kDa) was determined 3 days post-transduction. GFP protein was detected in all samples at different virus volumes (0.2, 0.5, 1.0, and 2.0 µL). Cell lysate from untransduced 293T was used as negative controls. Whole-cell lysate from pTATκ-GFP-transfected 293T cells (with prior confirmation of the GFP expression) was used as a positive control (~28 kDa). Tubulin (~55 kDa) was used as loading control for each sample

3. Boil the mixture at 95 °C for 5–10 min before loading the samples onto 10% SDS-polyacrylamide gel.
4. Run sample at 165 V for 35–45 min.
5. Transfer protein electrophoretically to Hybond ECL nitrocellulose membranes at 165 V for 2 h (*see* **Note 26**).
6. Block the membrane for 1 h in blocking buffer (Bb) (*see* **Note 27**).
7. Rinse the membrane three times in TBS-Tween 20 prior to addition of primary antibodies.
8. Incubate the membrane overnight with antibody buffer containing the primary antibody (1:1000 dilutions) at 4 °C (*see* **Note 28**).
9. The following day, rinse the membrane three times in TBS-Tween 20, and further expose to appropriate horseradish peroxidase-conjugated secondary antibodies at 4 °C for 1 h.
10. Rinse the membrane three times, and incubate with enhanced chemiluminescence reagent at room temperature for 5 min to detect immunoreactive bands.
11. Develop the film using a Photon Imaging system SRX-101A.
12. Develop the film in 30 s–1 min of developer/replenisher chemical, followed by 30 s–1 min of fixative/processing chemical, wash in $dH_2O$, and air-dry.
13. For future probing: Wrap the membrane in cling film, and store at −20 °C until needed (*see* **Note 29**). To re-probe the membrane with a different primary antibody, the previous antibody is first removed by stripping the membrane using 1× stripping buffer three times at 56 °C for 15 min. Rinse the membrane three times using TBS-Tween 20. Later, block the stripped membrane for 1 h in blocking buffer (*see* **Note 27**), and repeat **steps 8–11** for primary antibody probing as previously mentioned.

## 4 Notes

1. Add protease inhibitor cocktail in the last step. It contains enzymes to help protect the integrity of proteins during protein extraction and purification. If it is left too long at room temperature during the preparation of the LyB, it might degrade the enzyme function. It is also advisable to aliquot the LyB to preserve its function, and also can be kept for longer by minimizing the repeating freeze and thaw process.
2. The bovine albumin can also be used to substitute the skimmed milk powder as a part of blocking buffer components. Make sure that the fraction of this bovine albumin is fully dissolving in the buffer before using it.

3. Sodium azide is an inorganic compound that produces toxic gas. It is used as a preservative component in the antibody buffer to preserve the integrity, and function of the antibodies used in this case is for protein detection in western blot method.
4. The pH 6.96 of 2× HeBSS buffer is very crucial for the success of the plasmid transfection using the $CaPO_4$ chemical method. The efficiency of the transfection is affected by pH of the buffer. Therefore it is wise to optimize and adjust the pH as accurate as possible using the HCl.
5. In the case of in-house preparation of the SDS-PAGE gel, it is advisable to layer the top of the resolving gel buffer in the disposable cassette using absolute ethanol. This is to remove extra bubbles that form on the top and also to make sure that the edge top is even instead of crooked before layering with the stacking gel buffer.
6. Make sure to remove the ethanol by gently rinsing it a few times with water. Dry it with filter paper before adding the stacking gel buffer. Add the stacking gel buffer until it overloads the brim. This is to make sure that there are enough buffers when inserting and fixing the cassette comb.
7. The SDS-PAGE gel can be prepared in advance using the disposable cassette (or depends on the western blot system). This pre-cast gel can be kept in the 1× running buffer for at least 3 months.
8. Make sure that the ECL reagent is applied evenly on the membrane. Remove the excessive reagent by tapping the surface of the membrane with kimwipes or thin absorbent paper before applying the X-ray film. Make sure that it is done in the darkroom with safe lights to protect the X-ray film, and to prolong the ECL effect.
9. The standard molecular biology method with a slight modification is used to produce the vector expression plasmids encoding the gene marker (in this case is green fluorescent protein, GFP). This includes plasmid expansion and extraction using available commercial kits, PCR, enzymes digestion, etc..
10. It will take approximately 3 days to achieve at least 70–80% confluent of the 293T cells to grow in the 435 $cm^2$ triple-layer flasks. It is crucial for the $CaPO_4$ chemical transfection method, and to increase the virus titer. The reason is that some of the cells might die during the transfection process and thus it is wise to have the optimal number of the cells.
11. Important: Slowly, drop the mixture (one drop every second) to the 2× HeBSS. Do not do otherwise. This is to make sure that the plasmids precipitate evenly.

12. The incubation time for the coprecipitation of the plasmids is crucial as it will give enough time for the plasmid to form precipitation, and at the same time preserve the plasmid from degrading if it is left too long at room temperature. Thus, keep the incubation time within 20–30 min.
13. Mix well the DNA Ca-$PO_4$ coprecipitation mixture with the 80 mL complete media. Slowly and steadily pour the mixture down through the flask's bottle neck. This is to avoid the cell detached from the bottom of the flask as the 293T is considered fragile cells.
14. Let the mixture to evenly separate between the flask's compartments by 3–5-min vertical stand. This is to make sure that all bottom layer of the flask receive the equal volume of the mixture which will cover the whole surface.
15. When laying the flask horizontally, do it very quick but gently, so as not to detach the cells. If it is too slow, it might not cover the whole surface of the top compartment, as the mixture has slowly transferred to the lower bottom of the other compartment instead.
16. The fresh complete media must be replaced. The transfection media does decrease cell survival. Gently, replace the fresh media, to avoid cells from detaching.
17. Harvest the virus at 48 h, filter through 0.45 μM, and keep in the sterile contained at 4 °C (this procedure is repeated in the second harvest at 72 h). Replenish 80 mL of complete media.
18. Remove the remaining supernatant by putting the centrifuge container upside down for 5–10 min (do not exceed the time as it will dry the pellet).
19. Briefly, thaw the virus and discard the remaining lentiviral vector from the tube accordingly by following the hazardous protocol. Do not freeze the remaining lentiviral vector as it will degrade and will not be accurate for future transduction.
20. qRT-PCR method can be used to determine the lentiviral vector titer by quantitative detection of RNA genome copies of the envelope protein of the lentiviral vector. However, it does not reflect the lentiviral vector functionality. In some cases, it will be better to determine the titer by flow cytometry instead, especially for proteins that can be conjugated with fluorochrome antibodies, which is easy to measure.
21. We suggest starting at small volume: 0.2, 0.5, 1.0, and 2.0 μL (you may increase the volume accordingly). Four different lentiviral vector's volumes are sufficient to determine the titer. Always do the experiment in triplicate for statistical analysis.
22. Day 3 post-transduction is the optimal time to determine the lentiviral vector titer as it has been done for other lentiviral vectors.

Based on previous studies, the lentiviral vector enters target cells by day 3 post-transduction. However, in our previous experience, integrase-defective lentivirus will achieve the highest expression at day 3, and the expression will decrease over time (in our experiment almost zero expression by day 10 post-transduction) [21]. In wild-type SFFV-GFP-transduced cells, GFP expression is remained consistently at about 100%. Similar results were reported elsewhere, where the GFP expression dropped quickly within the first week after transduction [22–24]. Thus, the best time is at day 3 post-transduction for integrase-defective lentiviral vectors. It is advisable to plot graft for all data (triplicate). Choose the best lentiviral vector's volume that expresses 5–20% GFP, and calculate titer using the given formula.

23. It is important to know that different qPCR machines will have different settings. You might need to optimize accordingly using the protocol given by the manufacturer of the commercial kits used for the assay. Some commercial kits will advise different settings.
24. There are a few cationic polymers that can be used to enhance or increase the efficiency of transduction in certain cells. Polybrene acts by neutralizing the charge repulsion between virions and the cell surface. It can be toxic in some susceptible cells. Thus, it is recommended optimizing the concentration, so it will not kill the cells. Alternatively, DAEA-Dextran, which is less toxic and very effective for transient transfection, can be used as substitute.
25. Incubation must be carried out on ice, as the LyB contains protease cocktail inhibitors which need to be preserved at cold temperature. This will allow the protein's integrity from extracted cells to be well preserved.
26. Ideally, transfer the protein in cold room or keep the blot tank in container with ice. This is to avoid protein from degrading during the high voltage.
27. One hour is more than enough to block the unspecific protein on the membrane. However, it is also recommended to block the membrane overnight at 4 °C or cold room with continuous gentle shake.
28. In the case of detecting two or more different proteins with distance protein weight, both primary antibodies can be added together in the antibody buffer and incubate overnight at 4 °C. Alternatively, the second primary antibody can be detected by re-probing the membrane with special stripping buffer to remove the previous antibody and ECL reagent. Optimization of the concentration used to probe the protein is crucial to make sure that it binds the right protein, and to

eliminate high (or dark) background when developing the film later. It is also wise to use the monoclonal antibody instead of polyclonal to avoid the multiple bands due to truncated or unspecific proteins.

29. It is recommended to store at –20 °C for long-term analysis (can last up to 1 year) by wrapping using cling film. To re-probe, make sure that the frozen membrane is thawed at room temperature to avoid breakage before re-probe, or alternatively put the membrane in wash buffer to soften it. Membrane can also be kept in wash buffer if needed to re-probe within 1-month period.

## Acknowledgments

This work was supported by the Ministry of Higher Education of Malaysia and Universiti Kebangsaan Malaysia. The work has been carried out at the King's College London, Department of Haematological Medicine, The Rayne Institute, London, UK.
*Conflict of interest*: The authors declare no conflict of interest.

### References

1. Meissner A, Wernig M, Jaenisch R (2007) Direct reprogramming of genetically unmodified fibroblasts into pluripotent stem cells. Nat Biotechnol 25:1177–1181
2. Park IH, Zhao R, West JA et al (2007) Reprogramming of human somatic cells to pluripotency with defined factors. Nature 451:141–146
3. Wernig M, Meissner A, Foreman R et al (2007) In vitro reprogramming of fibroblasts into a pluripotent ES-cell-like state. Nature 448:318–324
4. Zufferey R, Dull T, Mandel RJ et al (1998) Self-inactivating lentivirus vector for safe and efficient in vivo gene delivery. J Virol 72:9873–9880
5. Wanisch K, Yanez-Munoz RJ (2009) Integration-defective lentiviral vectors: a slow coming of age. Mol Ther 17:1316–1332
6. Yamashita M, Emerman M (2006) Retroviral infection of non- dividing cells: old and new perspectives. Virology 344:88–93
7. Nightingale SJ, Hollis RP, Pepper KA et al (2006) Transient gene expression by nonintegrating lentiviral vectors. Mol Ther 13:1121–1132
8. Aiuti A, Biasco L, Scaramuzza S et al (2013) Lentiviral hematopoietic stem cell gene therapy in patients with Wiskott-Aldrich syndrome. Science 341:1233151
9. Lewinski MK, Bisgrove D, Shinn P et al (2005) Genome-wide analysis of chromosomal features repressing human immunodeficiency virus transcription. J Virol 79:6610–6619
10. Hacein-Bey-Abina S, Von Kalle C, Schmidt M et al (2003) A serious adverse event after successful gene therapy for X-linked severe combined immunodeficiency. N Engl J Med 348:255–256
11. Okita K, Matsumura Y, Sato Y et al (2011) A more efficient method to generate integration-free human iPS cells. Nat Methods 8:409–412
12. Vargas J, Gusella GL, Najfeld V et al (2004) Novel integrase-defective lentiviral episomal vectors for gene transfer. Hum Gene Ther 15:361–372
13. Peluffo H, Foster E, Ahmed SG et al (2013) Efficient gene expression from integration-defective lentiviral vectors in the spinal cord. Gene Ther 20:645–657
14. Apolonia L, Waddington SN, Fernandes C et al (2007) Stable gene transfer to muscle using non-integrating lentiviral vectors. Mol Ther 15:1947–1954
15. Leavitt A, Robles G, Alesandro N et al (1996) Human immunodeficiency virus type 1 integrase

mutants retain in vitro integrase activity yet fail to integrate viral DNA efficiently during infection. J Virol 70:721–728

16. Engelman A (1999) In vivo analysis of retroviral integrase structure and function. Adv Virus Res 52:411–426
17. Shawand A, Cornetta K (2014) Design and potential of non-integrating lentiviral vectors. Biomedicines 2:14–35
18. Bayer M, Kantor B, Cockrell A et al (2008) A large U3 deletion causes increased in vivo expression from a nonintegrating lentiviral vector. Mol Ther 16:1968–1976
19. Lombardo A, Genovese P, Beausejour CM et al (2007) Gene editing in human stem cells using zinc finger nucleases and integrase-defective lentiviral vector delivery. Nat Biotechnol 25:1298–1306
20. Kingston RE, Chen CA, Rose JK (2003) Calcium phosphate transfection. Curr Protoc Mol Biol. Chapter 9, Unit 9.1
21. Nordin F, Abdul Karim N, Wahid SFA (2014) Transgene expression is transient in non-integrating lentiviral-based transduction system: an alternative approach. Regen Res 3:1–7
22. Philippe S, Sarkis C, Barkats M et al (2006) Lentiviral vectors with a defective integrase allow efficient and sustained transgene expression in vitro and in vivo. Proc Natl Acad Sci U S A 103:17684–17689
23. Cornu TI, Cathomen T (2007) Targeted genome modifications using integrase-defective lentiviral vectors. Mol Ther 15:2107–2113
24. Sloan R, Wainberg M (2011) The role of unintegrated DNA in HIV infection. Retrovirology 8:52