Protocol for Lentiviral Transduction of Human Cells

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1. General Information

This protocol describes the standard technique for handling recombinant lentiviruses, as well as instructions on how to design an approach for transducing human cells. As an example, a transduction experiment of the human fibrosarcoma cell line HT-1080 with a recombinant lentivirus harboring LifeAct®-TagRFP transgene is described.

2. Background

Replication-deficient recombinant lentiviruses are widely used in research laboratories. This modified lentivirus is still able to infect cells. However, the essential genes for producing new viral particles, also known as virions, are no longer present.

Lentiviral vectors derived from the human immunodeficiency virus (HIV-1) have become major tools for gene delivery into mammalian cells. The most advantageous feature of lentiviral vectors is the ability to mediate efficient transduction, integration, and long-term expression into dividing and non-dividing cells, both *in vitro* and *in vivo*.

Lentiviral vectors have a broad host cell range that includes cell types such as neurons, lymphocytes, and macrophages. Previously, retrovirus vectors could not be used for these cell types. Moreover, lentiviral vectors have

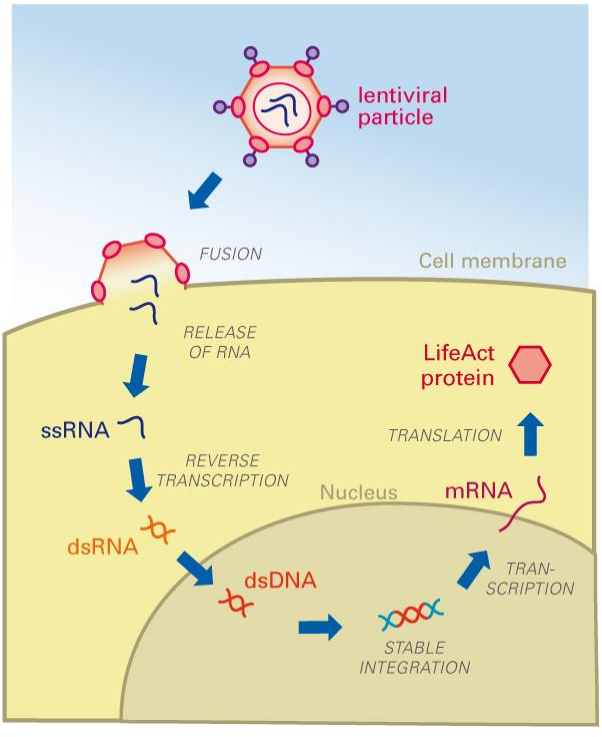

Figure 1: Simplified illustration of the lentiviral transduction mechanism.

proven to be effective in transducing brain, liver, muscle, and retina *in vivo* without any toxicity effects or immune responses.

The lentiviral particle binds to the cell membrane and enters the host cell. The viral RNA genome is released and reverse-transcribed to produce DNA. DNA is then stably integrated into the host genome at a random position by the viral integrase enzyme. A simplified illustration of the infection mechanism is shown in Figure 1.

3. Material and Equipment Required

For this protocol, the following materials are required:

Table 1: Materials needed for the transduction of HT-1080.

Name	Company	Order No.
rLV^{Ubi}-LifeAct®-TagRFP	ibidi GmbH	60141
µ-Dish $^{35mm, high}$ ibiTreat	ibidi GmbH	81156
HT-1080	commercially available	-
DMEM	Sigma-Aldrich Chemie GmbH	D5796
FBS/FCS	Sigma-Aldrich Chemie GmbH	F0804
Polybrene	Sigma-Aldrich Chemie GmbH	S2667
Puromycin	Life Technologies	A1113803

For this protocol the following equipment and instruments are required:

- Cell culture incubator (high humidity, 37°C, 5% CO_2)
- Class II biological safety cabinet
- Fluorescence microscope equipped with an appropriate filter set for TagRFP (fluorescence properties: Ex_{max} 555 nm / Em_{max} 584 nm), a stage top incubator (37°C, 5% CO2), and an optional time lapse function

4. Safety and Handling of Recombinant Lentiviruses

Avoid repeated thawing and freezing cycles during the performance of the experiments as this leads to a strong decrease in viral titer. Thawing should occur on ice, and the lentivirus should be used directly afterwards. Freezing should occur either on dry ice or at a temperature of -80°C.

In addition, to ensure that the quality of the virus is maintained, we recommend aliquoting the vial contents on first use.

Keep in mind that you will be working with infectious viral particles. Follow the recommended NIH guidelines for all materials containing BSL-2 organisms. The use of a Class II biological safety cabinet is mandatory. Work under a biosafety hood, use filtered tips, and wear gloves.

5. Transduction of HT-1080 with rLV^{Ubi}-LifeAct®-TagRFP

Basic terms

MOI: Multiplicity of Infection

TU: Transducing Units (also known as IP, IFU, or PFU)

rLV: recombinant Lentivirus

Experiments can be started once the MOI, one that is most suitable for the cells of interest, has been determined (see Section 7 for further information). In this example, HT-1080 cells cultured in a µ-Dish $^{35mm,\ high}$ ibiTreat have been used. As shown below, these cells can be efficiently transduced with a MOI of 10.

a. Calculating the amount of virus required in a µ-Dish $^{35mm,\ high}$

General formulas:

I. $$\text{virus needed}[TU] = \text{cell number seeded} * MOI$$

II. $$\frac{\text{virus needed}[TU]}{\text{viral titer}[TU/\mu l]} = \mu l \text{ needed}$$

Example:

I. Amount of virus needed for transduction:

   Cell number seeded = 3.5x 10^4 cells per µ-Dish; MOI = 10

   ⇨ Virus needed [TU]: 3.5x 10^4 (cells) * (MOI) 10 = 35x 10^4 TU per µ-Dish

II. Transforming the amount of virus into µl:

   Viral titer = 1x 10^7 TU/ml = 1x 10^4 TU/µl; Virus needed [TU] = 35x 10^4 TU

   ⇨ Virus needed [µl]: 35x 10^4 TU / 1x 10^4 TU/µl = 35 µl viral stock per µ-Dish.

As a result, 35 µl of viral stock (i.e., 1x 10^7 TU/ml) is needed to transduce 3.5x 10^4 cells with a MOI of 10.

Table 2: Number of cells and number of infectious lentiviral particles needed for setting up a lentiviral experiment with HT-1080 cells in other formats.

			Lentivirus amount [µl]					
Format	**HT-1080/ Well**	**Volume/ Well**	**MOI 1**	**MOI 2**	**MOI 5**	**MOI 10**	**MOI 15**	**MOI 30**
48 well	1x 10^4	250 µl	1	2	5	10	15	30
24 well	2x 10^4	500 µl	2	4	10	20	30	60
12 well	4x 10^4	1 ml	4	8	20	40	60	120
6 well	1x 10^5	2 ml	10	20	50	100	150	300
µ-Dish	3.5x 10^4	500 µl	3.5	7	17.5	35	52.5	105

b. Seeding cells

The day before the transduction, seed 3.5x 10^4 HT-1080 cells in 500 µl of cell growth medium into the inner well of the µ-Dish $^{35mm,\ high}$. After cell attachment add additionally 1.5 ml of growth medium. The cells should exhibit 20-30% confluence at the time of transduction.

c. Transduction

Note: If a cell type is transduced with a lentivirus for the first time it is recommended to perform a setup with different MOI using a lentivirus encoding a fluorescent protein. (see Table 2)

1. Thaw the lentivirus on ice.
2. Mix 8 µl Polybrene (1 mg/ml aliquot) with 957 µl culture.
3. Add 35 µl of lentiviral particles and mix the solution by flicking the tube. The final concentration of Polybrene is 8 µg/ml.
4. Remove culture medium from the cells, and add the Lentivirus/Polybrene mixture.
5. Optionally, perform a spinoculation step. Centrifuge for 90 min at 800x g, if you transduce difficult-to-transfect cells.
6. Incubate cells over night at standard cell culture conditions.
7. The next day, exchange Lentivirus/Polybrene mixture by fresh culture medium.
8. Incubate cells at standard cell culture conditions. The LifeAct®-TagRFP signal is visible after 3-4 days.
9. Image cells by fluorescence microscopy or perform another assay (e.g., flow cytometry).

d. Selection of stable cells

Note: Fluorescence intensities of stably transduced cells have to be monitored daily under the microscope. Usually, the maximal intensity of fluorescent signals is reached 4 - 6 days after transduction.

For selection of stable cells, puromycin needs to be added to the culture medium. For best results, puromycin needs to be titrated with nontransduced cells. Typical final concentrations range from 0.1-10 µg/ml. Replace the culture medium 48-72 hours after the transduction process with fresh puromycin-containing medium every 3-4 days until resistant colonies can be identified.

e. Results

Figure 2 below shows HT-1080 cells transduced with a MOI of 10 and imaged 72 hours after transduction. Almost 80% of the cells are expressing LifeAct®-TagRFP, which causes the bright staining of the actin cytoskeleton.

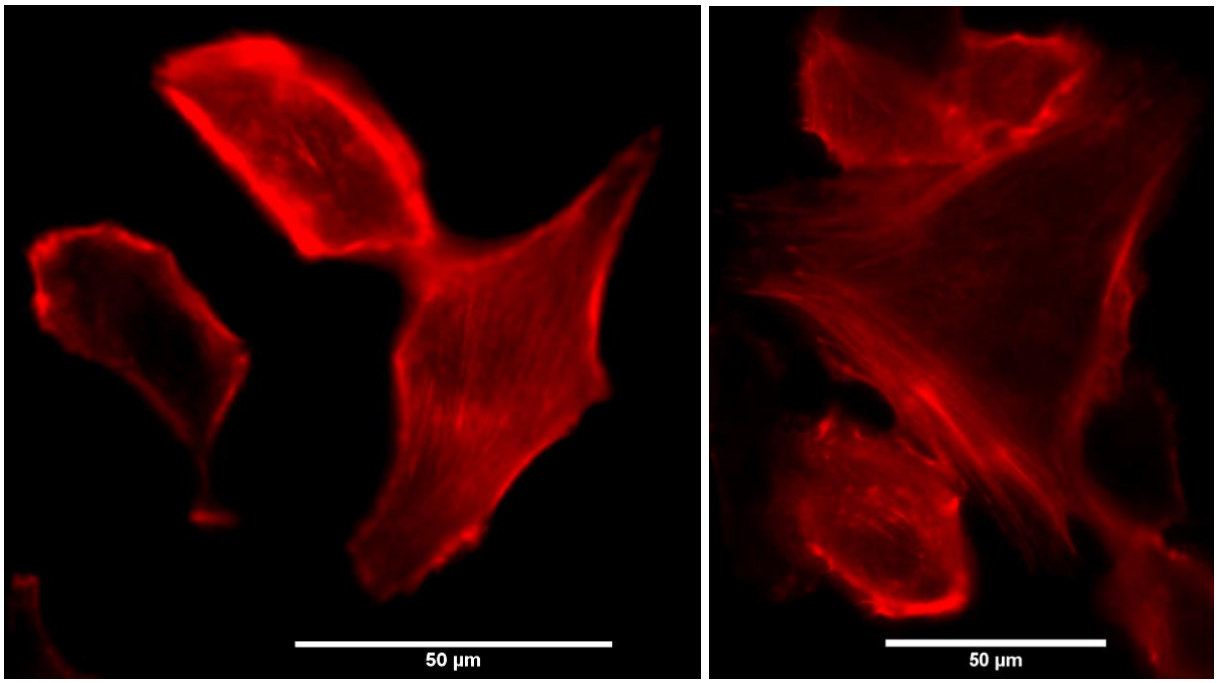

Figure 2: Fluorescence images of living HT-1080 cells (Passage 43, confluence 80%) transduced with the rLV^{Ubi}-LifeAct®-TagRFP and a MOI of 10. The images were taken 72 hours after transduction.

6. Troubleshooting

Problem	Reason	Solution
Low transduction efficiency	MOI used is too low.	Use higher amount of lentivirus.
	Viral titer is not the infectious titer.	Check infectious titer by transducing cells with serial dilutions of your RFP-expressing lentiviral vector. Repeat experiment with correct titer.
	Cells are very hard to transduce.	Increase the amount of lentivirus. Use LentiBoost (Sirion Biotech GmbH) instead of Polybrene. Use protocol with spinoculation step.
Low viability	Cells are sensitive to transduction enhancer.	Use LentiBoost (Sirion Biotech GmbH). Try Solution A and B of LentiBoost separately.
	Cells are sensitive to lentiviral treatment.	Use lower amount of lentivirus. Change medium 4 hours after transduction.

7. Optional: Determining the MOI

The MOI describes the number of virus particles needed to infect one cell. However, the probability of a cell infection is subject to the statistical Poisson distribution. For example, a MOI of 10 signifies that 10 virus particles are needed for one cell to be infected efficiently. In principle, the MOI for lentiviral particles ranges from 1 to 30.

General formula:

$$\mathrm{MOI} = \frac{\mathrm{volume(virus)} * \mathrm{concentration(virus)}}{\mathrm{volume(cells)} * \mathrm{concentration(cells)}}$$

The MOI differs greatly between various cell types. Therefore, when transducing cells for the first time, we recommend determining the MOI necessary for efficient transgene expression in the cells of interest before starting with specific approaches. Various recombinant viruses can be used for this purpose, such as adenoviruses that only harbor GFP (Green Fluorescent Protein) and those that harbor β-lactamase, as they can be easily quantified.

Experiment in a 24-well plate format:

1. One day prior to the transduction, seed the cells of interest into seven wells (e.g., 1x 10^5 cells per well), so that their confluence at the time of transduction is about 20-30%.
2. The next day, thaw the virus particles on ice and add them to the wells, referring to the numbers in Table 2. To establish a control, leave one well free of virus particles. For easier handling, the virus can be diluted with 1x PBS or medium and pipetted in higher quantities into the wells. The quantity of the required diluted virus will depend on the level of dilution.
3. Incubate the cells for 72 hours at 37°C under standard cell culture conditions.
4. Acquire pictures of the cells by fluorescence microscopy after 24, 48 and 72 hours.

Analysis:

5. Determine the rate of transduced cells per well for each MOI and at each time point.
6. The lowest MOI at which all cells (or enough cells for the specific approach) show transgene expression is then used for further experimentation.

Note: High quantities of the virus could also lead to cytotoxic side effects. Therefore, in some cases it may be better to choose a lower MOI, so as to avoid artifacts due to non-healthy cells.

7. If needed, the MOI can be adjusted and further refined.

Figure 3 below illustrates possible results form testing different MOI on the cells of interest. In this example, an MOI of 10 was necessary to efficiently transduce 100% of the cells.

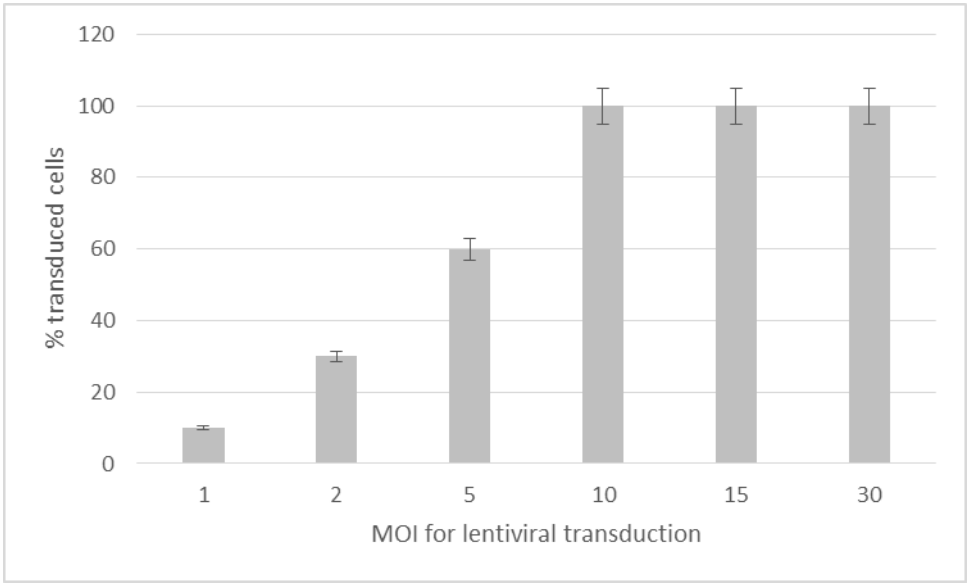

Figure 3: Percentage of transduced cells after transduction with different MOI.