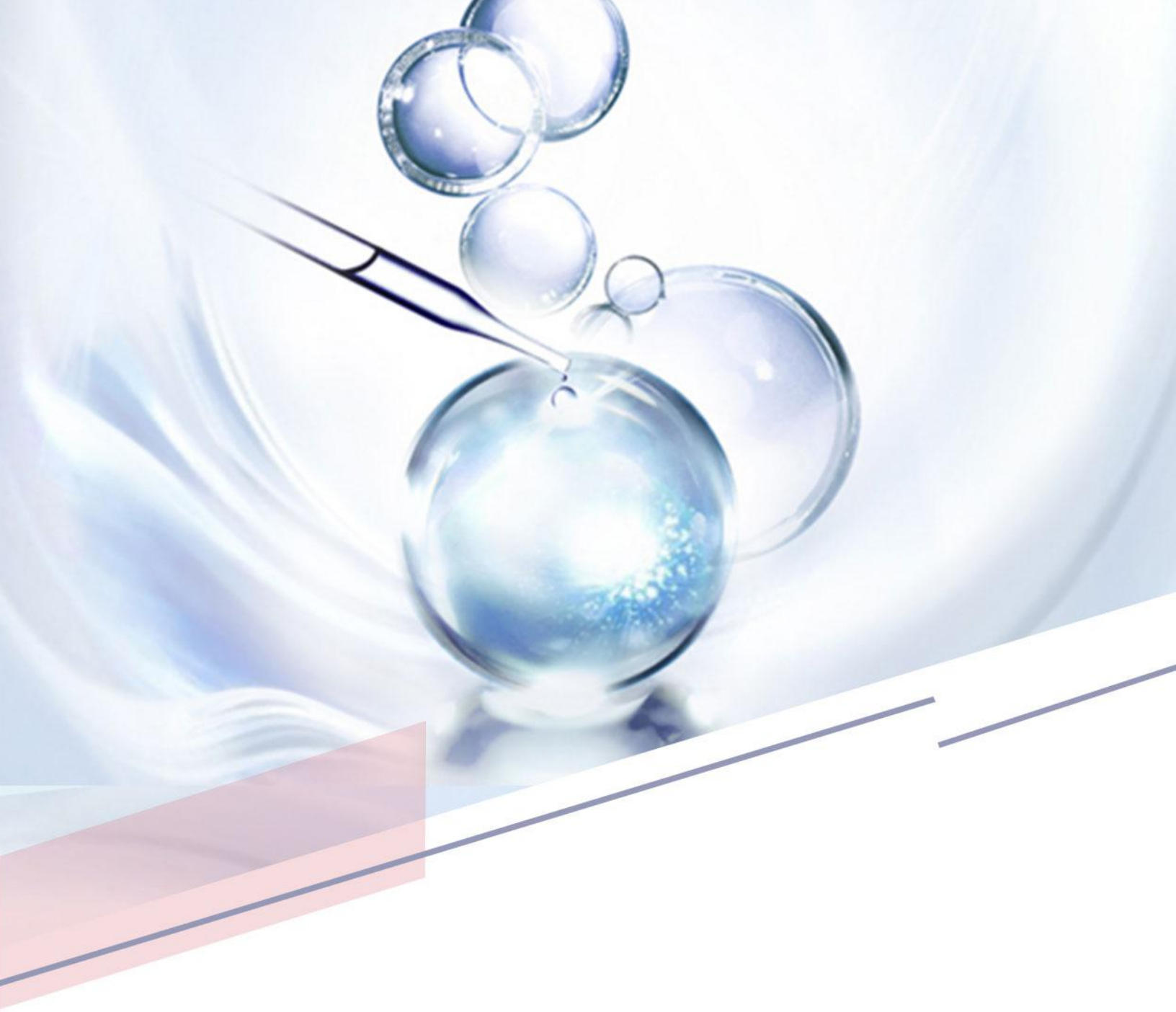

Lentivirus Production Protocol

Packaging Concentration and Purification

GENEMEDI
Innovation In Gene Therapy

Production protocol

Lentivirus

Safe Use of Lentivirus (Lv)

1. Lentivirus (Lv) related experiments should be conducted in biosafety level 2 facilities (BL-2 level).

2. Please equip with lab coat, mask, gloves completely, and try your best to avoid exposing hand and arm.

3. Be careful of splashing virus suspension. If biosafety cabinet is contaminated with virus during operation, scrub the table-board with solution comprising 70% alcohol and 1% SDS immediately. All tips, tubes, culture plates, medium contacting virus must be soaked in chlorine-containing disinfectant before disposal.

4. If centrifuging is required, a centrifuge tube should be tightly sealed. Seal the tube with parafilm before centrifuging if condition allowed.

5. Lentivirus related animal experiments should also be conducted in BL-2 level.

6. Lentivirus associated waste materials need to be specially collected and autoclaved before disposal.

7. Wash hands with sanitizer after experiment.

Storage and Dilution of Lentivirus

Storage of Lentivirus

Virus can be stored at 4°C for a short time (less than a week) before using after reception. Since Lentiviruses are sensitive to freeze-thawing and the titer drops with repeated freeze-thawing, aliquot viral stock should be stored at -80°C freezer immediately upon arrival for long-term usage. While virus titer redetection is suggested before using if the lentiviruses have been stored for more than 12 months.

Dilution of Lentivirus

Dissolve virus in ice water if virus dilution is required. After dissolving, mix the virus with medium, sterile PBS or normal saline solution, keeping at 4°C (using within a week).

Precautions

- Avoid lentivirus exposure to environmental extremes (pH, chelating agents like EDTA, temperature, organic solvents, protein denaturants, strong detergents, etc.)

- Avoid introducing air into the lentivirus samples during vortex, blowing bubbles or similar operations, which may result in protein denaturation.

- Avoid repeated freezing and thawing.

- Avoid exposing to “regular” plastics (especially polystyrene or hydrophobic plastics) for prolonged periods in liquid phase. Most lentivirus viruses are very sticky and loss can occur if exposed to regular plastics, including tubes, cell culture plates, pipette tips, if not frozen. It is best to store lentivirus in siliconized or low protein binding tubes. Pluronic F-68 used at 0.01%-0.1% in the formulation buffer will minimize sticking if regular plastics are used.

- Avoid diluting lentivirus into low salt solution. Some lentiviruses aggregate in low salt solution, which will be non-infectious.

Introduction of Lentivirus

Lentivirus (lente-, Latin for "slow") is a genus of retroviruses, causing chronic and deadly diseases by long incubation periods in human or other mammalian species [1]. To date, 5 serogroups have been recognized according to the vertebrate hosts they are associated with (primates, sheep and goats, horses, domestic cats, and cattle). Among them, the primate lentiviruses are distinguished by the utilization of CD4 surface protein as a receptor and the absence of dUTPase [2]. Derived from HIV-1, lentiviruses can integrate a significant amount of viral cDNA into the host genome, mediate stable and long-term transgene expression, and efficiently infect dividing cells and nondividing cells, which makes lentivirus an attractive gene delivery vehicle in most cell types [3].

Considering the key safety concerns during the use of HIV-derived lentivirus vectors, recombinant lentivirus has been designed and widely used for gene delivery in most cell types. As a research tool used to introduce a gene product into *in vitro* systems or animal models, lentiviral vector has been put into large-scale efforts to down-regulate or up-regulate gene expression in high-throughput formats, allowing researchers to examine the necessity and effects of transgenes in disease model systems, which is an indispensable for the discovery of novel transgenic drugs.

Nowadays, several generations of lentivirus packaging system are developed, in which the second-generation lentivirus vector and the third-generation lentivirus vector are the two most popular ones. The current method of the recombinant lentivirus production in Genemedi is based on three plasmids co-transfection system, involving the co-transfection of 3 plasmids (lentivirus series plasmid containing gene of interest (GOI) pLv-GOI, envelope expressing plasmid pMD2G and packaging plasmid pSPAX2) into 293T cells to generate lentivirus vectors.

Protocol Overview

A schematic overview of recombinant lentivirus production is shown in Figure 1. The first step is to clone the gene of interest (GOI) into an appropriate LTR/MCS containing vectors.

The recombinant expression plasmid is co-transfected into the 293T cells with envelope expressing plasmid pMD2G and packaging plasmid pSPAX2, which together supply all of the trans-acting factors required for lentivirus replication and packaging in the 293T cells. Recombinant lentivirus particles are prepared from infected 293T cells and may then be used to infect a variety of mammalian cells.

Upon infection of the host cell, virus genome ssRNA should be converted into double-stranded DNA in order for gene expression and virus replication. Together with other viral proteins, the newly synthesized DNA constitutes an integration-competent nucleoprotein complex, migrating into host cell nucleus and mediating integration of viral DNA into host chromatin. Integrated viral DNA, named as provirus, becomes part of host genome and serves as a transcription template for the synthesis of viral mRNA and genomic RNA. Following the synthesis of viral genomes and proteins, the viral components are assembled to produce new virions, the virus particles then bud out of host cell and undergo a maturation step to generate infectious lentivirus.

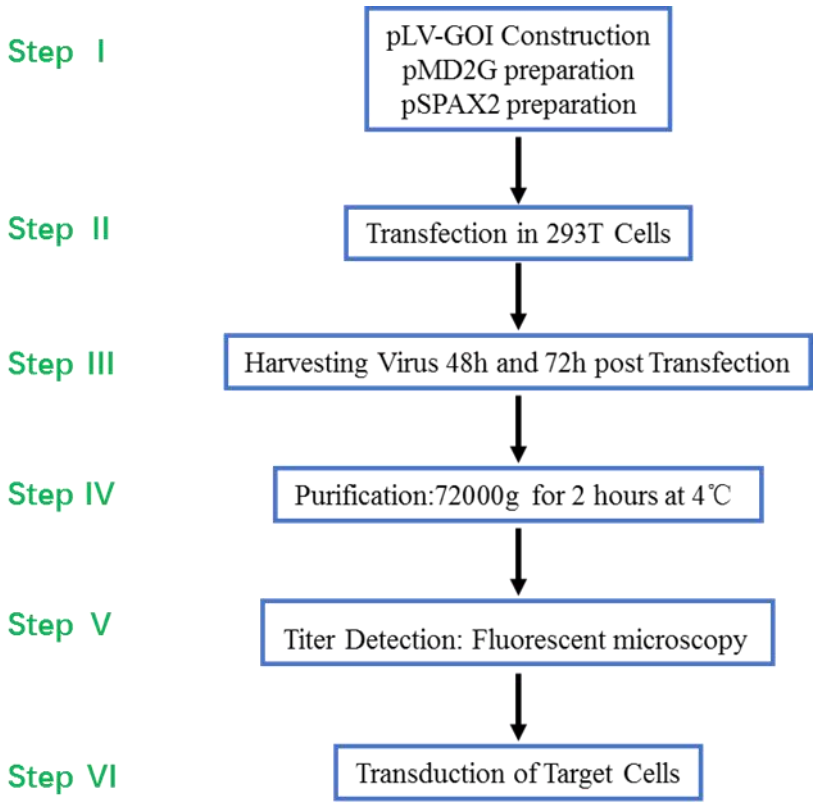

Figure 1. Lentivirus packaging experiment flow chart.

Experimental Materials

GeneMedi's lentivirus Vector System

GeneMedi's lentivirus Vector System, also named the lentivirus expression system or lentivirus packaging plasmid system, is a powerful tool for in-vitro & in-vivo gene delivery, shRNA mediated RNA interference (RNAi), gene editing and stable cellline development. You can easily produce a recombinant lentivirus particle in HEK293T or HEK293FT cell line in high titer using GeneMedi's lentivirus Vector System.

The Genemedi lentivirus vector system including 3 lentivirus packaging plasmids: the lentivirus expression plasmids with different promoters and tags, an envelope protein VSV-G expressing plasmid pMD2G and a packaging plasmid pSPAX2 expressing Gag-pol and Tat.

GeneMedi has developed a variety of lentivirus expression vectors with different expression cassettes, containing kinds of verified promoters and reporters including GFP, zsgreen, RFP, mcherry and luciferase. The GeneMedi's lentivirus expression vectors have been proved very suitable for unique gene overexpression or shRNA-mediated knock-down (also called RNAi (RNA interference). You can also achieve gene knock-out(KO) or gene editing using our Crispr-cas9-gRNA lentivirus expression vector.

Bacterium Strain

E.coli strain DH5α is used for amplification of lentivirus vector system plasmids.

Packaging Cell Line

293T is the virus packaging cell line that can facilitate initial production, titer detection of lentivirus. It is an epithelial-like cell line required for lentivirus replication, and grows into a monolayer when confluent.

The complete growth medium of 293T is Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 1% Penicillin-Streptomycin (Pen-Strep). For a continuous culture, cells should not exceed 70% confluence to maintain proper characteristics. Usually, starting from cell passage number one, optimal results can be obtained within 30 passages. Once reached, it is best to start a new culture from another frozen stock in case of any unexpected mutations and unhealthy growth. Therefore, banking your own 293T frozen stocks is very important to ensure experimental integrity and continuity. Freezing cells at the logarithmic phase will improve post-thaw viability.

Note: To maintain cells in a healthier condition and improve production efficiency of lentivirus, it is recommended to use our Genemedi anti-mycoplasma reagent CurePlasma™.

Other Materials and Reagents

Gene of interest
LB broth
Agar and Agarose
Kanamycin
Ampicillin
70 and 100% ethanol
Sterile PBS
Chlorine bleach
DNA gel apparatus and power supplies
Class II Biosafety Cabinet
37℃ orbital shaker
37℃ bacteria incubator
37℃, 5% CO_2 incubator
15- and 50-ml conical tubes
25- and 75-cm^2 tissue culture flasks
Ultracentrifuge (Beckman) or equivalent with SW28 rotor
Low-speed swinging-bucket centrifuge
Microcentrifuge
Centrifuge tube (thick-wall polycarbonate tube with cap)

Packaging and Concentration of Lentivirus

Vector Construction of Lentivirus

Before lentivirus packaging, the gene of interest should be constructed into lentivirus vector. Genemedi has plenty of premade lentivirus vector goods carrying some genetic tools in stock, such as lentivirus-LC3 autophagy flux detection biosensors, etc.

Click here to find more about Genemedi's ORF/CDNA premade lentivirus expression vectors

Note:

In order to construct vectors quickly and efficiently, it is strongly recommended to use Genemedi- ClonEasy™ One Step Cloning Kit.

Click here to find more about Genemedi - ClonEasy™ One Step Cloning Kit

Transfection of Virus Plasmids into 293T Packaging Cells

a. Propagate 293T cells in DMEM with 10% FBS and 1% pen/strep. The day before transfection, plate the cells in a 10cm dish such that the cells reach 70-80% confluency the next day. On the day of transfection, set up the 3-plasmid co-transfection as table 1.
b. DMEM needs to be preheated to 37℃ with water bath. LipoGene™ transfection reagent needs to be warmed up to room temperature before use, and mix gently before use. Replace the transfection medium of 10cm dish with fresh medium 6 hours after transfection.
c. To prepare viral plasmids for each reaction using a 60-mm dish:

Table 1. Plasmid and transfection reagent required for transfection.

Component	Amount
pLv-GOI vector	10μg
pMD2G	5μg
pSPAX2	10μg
LipoGene™	100μl

d. Mix plasmids with transfection reagent in DMEM and add drop-wise to pre-seeded 293T cells. Incubate at 37℃, 5% CO2 and refresh with complete culture medium in 6 hours.

Note:

1. *A detailed protocol of the transfection reagent can be referred to Genemedi LipoGene™ Transfection Reagent User Manual.*
   Click here to find more about Genemedi - LipoGene™ Transfection Reagent

2. *Cells should be in a healthy growth state for use prior to transfection.*

Harvest Virus

Collect the supernatant containing lentivirus particles 48 hours and 72 hours later after transfection, respectively. Replace fresh DMEM culture medium after the collection of supernatant at 48h.

Virus Purification

a. After collecting virus twice, discard the transfected 293T cells and filter the collected supernatant with 0.45μM filter membrane to an ultracentrifuge tube.

b. Centrifuge at 72000g for 2 hours at 4℃.

c. Then discard the supernatants and resuspend the lentivirus deposition with 500 μl fresh medium and keep at -80℃ or in liquid nitrogen for long time storage.

Titration of Purified lentivirus

Determine lentivirus titer with fluorescent microscopy.

a. Seed 293T 1×10^4 cells/well in a 96-well plate one day in advance.

b. Perform gradient dilution of the lentiviral particles to 1:10, 1:100, 1:1000, 1:10^4, 1:10^5, 1:10^6 in 100ul final volume in culture medium.

c. Total of 100μl viral particle mixture should be added to each well with at least 3 replicates per virus.

d. Two days post infection, count the fluorescent positive cells using fluorescent microscopy and select the dilution factor with a proper fluorescent positive proportion (10%-30% positive cells/well). Count the triplicates and average the number of positive cells.

e. Estimate the lentivirus titer using the following formulation: Viral titer (TU/ml) = number of fluorescent positive cells × 10 × dilution.

Transduction of Target Cells

This protocol is for the stable cell line construction based on puromycin selection.

Note:

MOI: multiplicity of infection, is the number of viral particles to infect one cell. An optimization test of MOI is strongly recommended as the real MOI to certain cells may be affected by the operations and methods of dealing with viruses in different labs.

Cell Preparation

Plate robust target cells into 24-well plates at a density of 1 x 10^5/ml one day in advance.

Note:

The number of planted cells depends on the growth rate of the relevant cell line. 50% to 70% confluence should be reached on the following day.

MOI Test of Lentivirus

a. Prepare the virus in 10-fold dilution gradient, and ensure the MOI is within a range of 3 to 1000.

b. Day 0: Plate target cells in good condition at a density of 1 × 10^5 /ml into 96-well plates, 100 µl per well. Incubate at 37℃ overnight.

c. Day 1: Prepare virus in a six-MOI gradient, and dilute proper amount of virus suspension in complete culture medium of target cell to a final volume of 100 µl (setting MOI = 3, 10, 30, 100, 300, 1000). Add diluted viruses to pre-seeded cells and incubate for 4 to 8 hours at 37℃, then refresh the medium to remove viruses.

d. Day 3: Detect fluorescence with a microscope. Calculate MOI based on the ratio of fluorescent cells.

Note:

If the virus is not fluorescence-labeled, MOI can be determined by qPCR, WB, IF, IHC, etc.

Table 2. The lentiviral MOIs of commonly used cell lines.

Cell line	MOI range	Auxiliary infection reagent polybrene
K562	20～40	Need
Jurkat	50～80	No
kasumi	10～30	No
NB4	50～80	No
U937	20～40	Need
THP-1	50～80	Need
GBC-SD	30～50	No
H929	100～150	No
H1299	1～3	Need
95D	2～4	Need
A549	20～40	Need
SPC-A-1	100～150	Need
7402	10～15	Need
Hep 3B	10～30	Need
Hep G2	10～30	Need
SMMC-7721	10～30	Need
Huh-7	10～30	Need
Hela	10～30	Need
HOS	20～40	Need

Hep-2	10～30	Need
HL-60	>100	Need
HT-29	10～30	Need
PKO	2～4	Need
SW480	10～30	Need
DLD-1	10～30	Need
SK-OV-3	2～4	Need
SHG-44	10～30	Need
U251	1～3	Need
U87	1～3	Need
293T	1～3	Need
HUVEC-2C	10～30	Need
PC-3	20～40	Need
MDA-MB-	10～30	Need
MCF-7	20～40	No
Tca8113	10～30	Need
RPE	10～30	Need
AGS	100～150	Need
BGC-823	100～150	Need
SGC-7901	10～30	Need
MKN-28	20～40	Need
MKN-45	20～40	Need
BxPc-3	20～40	Need
CFPAC-1	50～80	Need
Panc-1	2～4	Need
HEC-1-B	2～4	Need
NIH-3T3	20～40	Need
Raw264.7	10～30	No
CHO	20～40	Need
HSC-T6	10～30	No
C6	>100	Need
NRK	10～30	Need

Transduction

a. Before infection, virus should be melted on ice gently and resuspended in culture medium.

b. Prepare the virus in 10-fold dilution gradient, and ensure the MOI is within a range of 3 to 1000.

c. Remove the preceding medium and add lentivirus-containing medium with 1/2 volume of normal culture volume.

d. Culture for 4 hours at 37℃, and supplement fresh medium to normal volume. The recommended medium volume of lentivirus infection is displayed in the following table 3.

e. Refresh the culture medium 24 hours post infection.

Table 3. The recommended medium volume during lentivirus infection.

Culture	Surface	Normal volume for cell	1/2 Volume for lentivirus
96-well	0.3 cm^2	100 μl	50 μl
24-well	2 cm^2	500 μl	250 μl
12-well	4 cm^2	1 ml	500 μl
6-well	10 cm^2	2 ml	1 ml

Construction of Stable Transgenic Cell Lines

48 hours post infection, change to fresh medium with puromycin. The recommended concentration of puromycin ranges from 1 to 10 μg/ml according to cell lines. Set the uninfected wild-type cells as control group and add equal volume and concentration of puromycin. Replace with fresh puromycin-containing medium every 2 or 3 days until the control group cells die out. Then choose one of the following steps according to experimental requirements.

a. Non-selecting monoclonal cells

Passage the infected cells and select with puromycin constantly. Freeze the cell mixture in continuous three passages. Considering the heterogeneity, we recommend selecting monoclonal cell for a confirmed phenotype.

b. Selecting monoclonal cells

Select at least five monoclonal cells after infection and puromycin selection, and propagate in puromycin-containing medium. Detection the expression of target genes using western blot or qPCR. Choose the stable cell line with proper expression level of target genes to passage three generations and freeze the stable cell line.

Notes for infection of special cell lines:

1. Suspension cells

We recommend using flat fillet centrifuging transfection to infect suspension cells or semi-suspension cells. Add virus suspension into cell culture dish, sealing tightly, and centrifuge at low speed of 200g for 1 hour in the flat fillet centrifuge. Place cells in cell culture incubator after centrifuging transfection. If the flat fillet centrifuge is inaccessible, you can suspend the cells and transfer cells into centrifuge tubes, followed by low-speed centrifuge, and discard the most of supernatant. Add virus suspension into the tubes, resuspending cells, place it at room temperature for 15 min (no more than 30 min), and transfer the cells and virus suspension into plate to culture. Replace with fresh culture medium the next day.

2. Cells difficult to infect

For cells difficult to infect, like DC cells, we recommend repeated infections. Replace with fresh virus suspension 24 hours after the first infection. Repeated infections can increase the infection efficiency markedly.

3. Non-dividing primary cells

We recommend high-titer adenovirus to infect these cells like BMSC.

References

1. Salgado CD and JM Kilby. (2009). Retroviruses and other latent viruses: the deadliest of pathogens are not necessarily the best candidates for bioterrorism. J S C Med Assoc 105:104-6.

2. Piguet V, O Schwartz, S Le Gall and D Trono. (1999). The downregulation of CD4 and MHC-I by primate lentiviruses: a paradigm for the modulation of cell surface receptors. Immunol Rev 168:51-63.

3. Cockrell AS and T Kafri. (2007). Gene delivery by lentivirus vectors. Mol Biotechnol 36:184-204.

Related products

1. AAV packaging system

AAV1 vector system	AAV6 vector system	AAV-DJ vector system
AAV2 vector system	AAV8 vector system	AAV-Dj8 vector system
AAV2 variant(Y444F) vector system	AAV8 variant (Y733F) vector system	AAV2-Retro (Retrograde) vector system
AAV2 variant (Y272F,Y444F,Y500F,Y730F) vector system	AAV8 variant(Y733F, Y447F) vector system	AAV9-PHP.B vector system
AAV2 variant(Y444F,Y730F,Y500F,Y272F,Y704F,Y252F) vector system	AAV8 variant(Y733F,Y447F,Y275F) vector system	AAV9-PHP.eB vector system
AAV2 variant(AAV2.7m8) vector system	AAV9 vector system	AAV9-PHP.S vector system
AAV5 vector system	AAV-Rh10 vector system	

More details please visit: https://www.genemedi.net/i/aav-vector-system

2. Lentivirus Promise-ORF™ :

Sequence-verified CDNA clones in lentivirus and mammalian expression vectors.

More details please visit: https://www.genemedi.net/l/lv-plasmid

3. Lentivirus custom production

More details please visit: https://www.genemedi.net/i/lentivirus-custom-packaging

Other protocols

· Adeno-associated Virus (AAV) Production Protocols:
https://www.genemedi.net/pdf/AAV%20production%20protocol-packaging%20concentration%20and%20purification-GeneMedi.pdf

· Adenovirus Production Protocols: https://www.genemedi.net/pdf/Adenovirus%20production%20protocol-packaging%20concentration%20and%20purification-GeneMedi.pdf

· Crispr/cas9 mediated Gene knockout in Mammalian Cells - Protocol
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· CRISPR/Cas9 AAV Production - User Manual
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· Recombinant Adenovirus-CRISPR/Cas9 Knockout System-User Manual
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· Recombinant Lentivirus-CRISPR/Cas9 Knockout System-User Manual
https://www.genemedi.net/pdf/Genemedi-Lentivirus-crispr%20User%20Manual.pdf

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