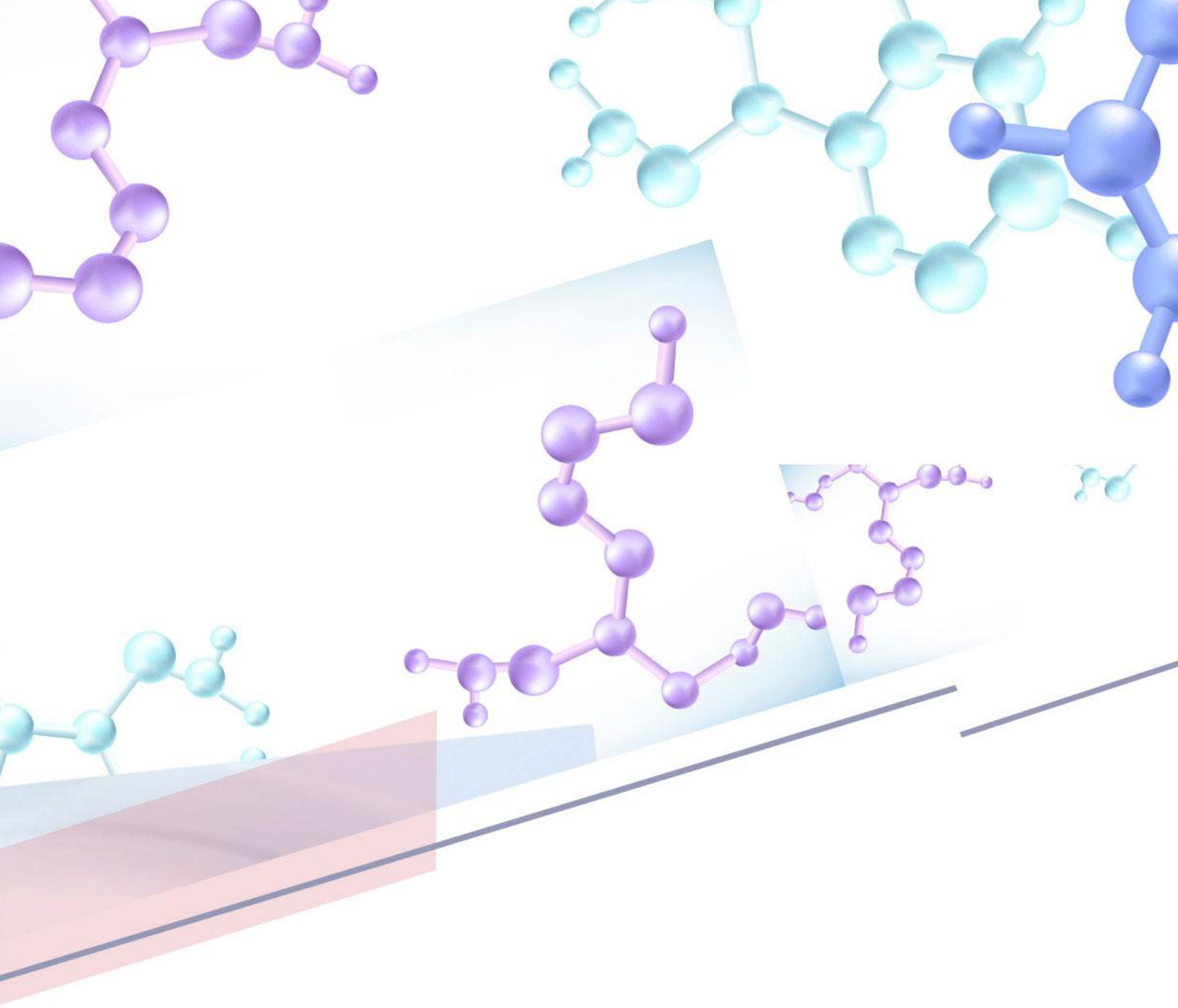

Adenovirus Production Protocol

Packaging Concentration and Purification

GENEMEDI
Innovation In Gene Therapy

Production protocol

adenovirus (AdV)

Introduction of Recombinant adenovirus (AdV)

Recombinant adenovirus (rAdV) is a replication-defective adenoviral vector, which is widely used for a variety of purposes including gene transfer and engineering, vaccination and gene therapy [1,2]. There are several advantages of using adenovirus as a gene transfer mediator. Firstly, it can deliver as large as 8 kilo-base (kb) gene sequences into cells and tissues without insertion of exogenous fragment in the genome. Secondly, almost all the dividing and non-dividing cells, primary cells and organ tissues can be transduced by recombinant adenovirus. Moreover, recombinant adenovirus is easy to operate and expand into large-scale, and the efficiency can reach up to 100%. Thus, recombinant adenovirus plays an important role in gene engineering research and potential therapeutic treatment of diseases.

The most commonly used adenovirus is serotype 5 (Ad5) of Homo Sapiens consisting of a double-stranded linear DNA molecule at about 36 kb in size1. The cytoplasmic membrane receptors and fibers facilitate endocytosis of adenovirus into cell cytoplasm, where adenovirus particles further migrate into cell nucleus for self-replication using replication machinery of the host [3]. Once replicated, the adenovirus genome is assembled into its protein shell and released from cells, causing cell lysis [3].

Nowadays, several packaging systems of recombinant adenovirus are developed, in which AdEasy1 and AdMAX2 are the two most popular ones, sharing a common strategy that target gene sequence is cloned into an adenovirus expression vector (adenovirus shuttle vector), then recombined into a viral backbone vector. Early viral transcription units, E1 and E3, are defected in both of these two systems, while E3 gene is not necessary for adenovirus replication1. Thus, packaging of recombinant adenovirus is usually conducted in cell lines expressing E1 gene, such as HEK-293, HEK-293A, etc. [2].

In comparison with AdEasy, AdMAX system is relatively easy to handle and can achieve higher adenovirus titer during adenovirus production. This recombinant adenovirus protocol is developed according to AdMAX system, using a two-vector system composed of adenovirus expression plasmids (adenovirus shuttle vectors) with different promoters and tags, and an adenovirus genome backbone plasmid with E1 region deletion.

Protocol Overview

A schematic overview of recombinant adenovirus (AdV) production is shown in Figure 1. The first step is to clone the gene of interest (GOI) into an appropriate plasmid vector. For most applications, the cDNA of interest is cloned into one of the recombinant adenovirus expression vectors. The inverted terminal repeat (ITR) sequences present in these vectors provide all of the cis-acting elements necessary for recombinant adenovirus replication and packaging.

The recombinant expression plasmid is co-transfected into the 293A cells (an E1-complementing cell line) with adenovirus genome backbone plasmid, which together supply all of the trans-acting factors required for adenovirus replication and packaging.

Small plaques can be visualized under microscope 10 to 21 days post-transfection. Pick three to six individual plaques and compare their adenovirus titer, then select the one with highest titer to proceeded subsequent amplification, concentration and purification experiments.

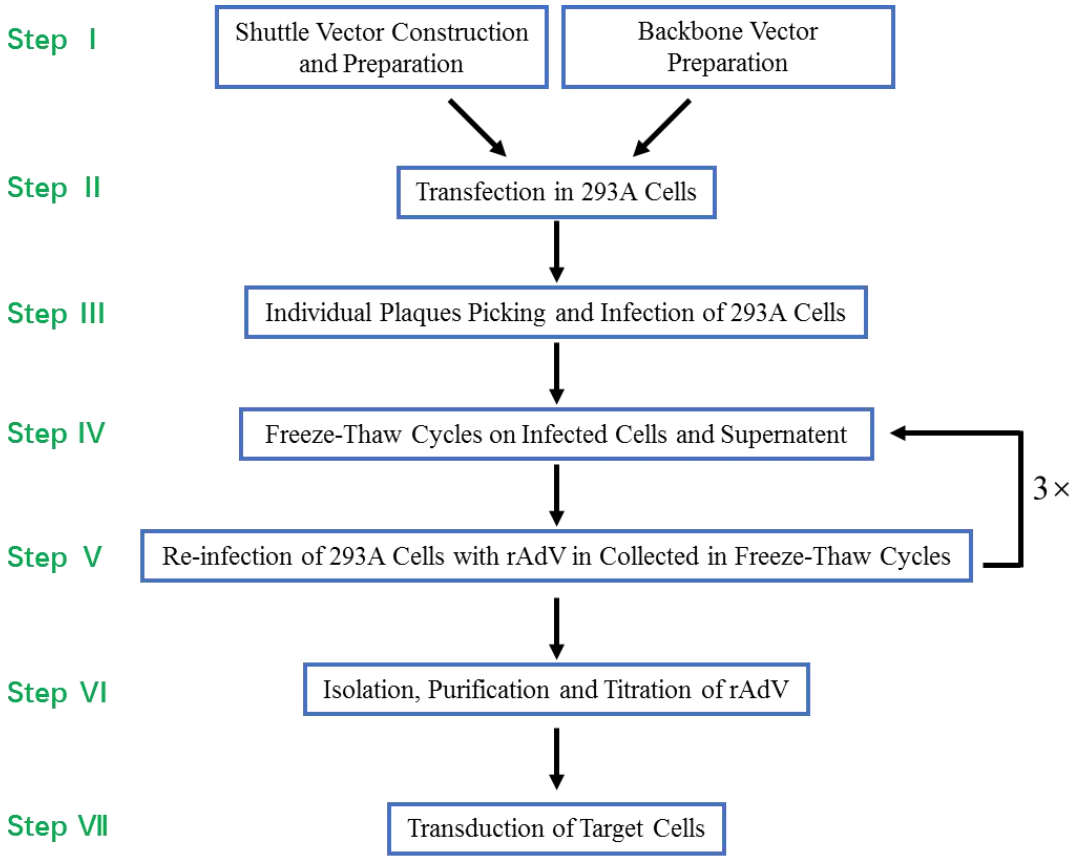

Figure 1. Adenovirus packaging experiment flow chart.

Experimental Materials

GeneMedi's adenovirus Vector System

GeneMedi's adenovirus Vector System, also named the adenovirus expression system or adenovirus packaging plasmid system, is a powerful tool for in-vitro & in-vivo gene delivery, shRNA mediated RNA interference (RNAi) and gene editing. You can easily recombine produce a recombinant adenovirus (r-AdV) particle in HEK293 or HEK293A cell line in high titer using GeneMedi's adenovirus Vector System.

The Genemedi adenovirus vector system including 2 types of adenovirus packaging plasmids: the adenovirus expression plasmids (adenovirus shuttle vectors) with different promoters and tags, and an adenovirus genome backbone plasmid with E1 region deletion.

GeneMedi has developed a variety of adenovirus expression vectors with different expression cassettes, containing kinds of verified promoters and reporters including GFP, zsgreen, RFP, mcherry and luciferase. The GeneMedi's adenovirus 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 adenovirus expression vector.

Visit https://www.genemedi.net/i/adenovirus-vector-system for more information about GeneMedi's adenovirus vector system and multiple adenovirus expression vectors (adenovirus shuttle vectors).

Bacterium Strain

E. coli strain DH5α is used for amplification of adenovirus expression plasmids (adenovirus shuttle vectors) and adenovirus backbone vector.

Packaging Cell Line

293A is the adenovirus packaging cell line that can facilitate initial production, amplification and titer determination of recombinant adenovirus. It is an adherent, epithelial-like cell line expressing E1 proteins required for adenovirus replication, and grows into a monolayer when confluent. Originated from the 293 cell line and established for plaque assays, this cell line was identified to be an easy-to-handle transfection host.

The complete growth medium of 293A 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 293A frozen stocks is very important to ensure experimental integrity and continuity. Freezing cells at the logarithmic phase will improve post-thaw viability.

Notices:

If the cell line is contaminated by mycoplasma, to reach a better-cultured cell state, we recommend the use of Genemedi anti-mycoplasma reagent CurePlasma™.

Packaging Cell Line

Gene of interest
LB broth
Agar and Agarose
Kanamycin
Ampicillin
70 and 100% ethanol
Sterile PBS
Cesium chloride (CsCl)
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
Cell scrapers
Dry-ice/methanol bath
Liquid nitrogen tank
Ultracentrifuge (Beckman) or equivalent with SW28 rotor
Low-speed swinging-bucket centrifuge
Microcentrifuge
Centrifuge tube (thick-wall polycarbonate tube with cap)
Ring stand and clamp, 3-ml syringes and 18-G needles

Packaging and Concentration of Adenovirus

Vector Construction of Adenovirus

Before recombinant adenovirus packaging, the gene of interest should be constructed into recombinant adenovirus expression vector. Genemedi also provides various Adenovirus vectors with alternative promoters and fluorescent labels (Table 1). What's more, Genemedi has plenty of premade Adenovirus vector goods carrying some genetic tools in stock, such as adenovirus-LC3 autophagy flux detection biosensors, etc.

Visit https://www.genemedi.net/i/virus-plasmid to find more about *Genemedi's ORF/CDNA premade adenovirus expression vectors*

Note:

In order to construct vectors quickly and efficiently, it is strongly recommended to use Genemedi- ClonEasy™ One Step Cloning Kit（Cat. GM-GC-01/02/03）.

Visit https://www.genemedi.net/i/cloneasy-one-step-cloning-kit to find more about *Genemedi - ClonEasy™ One Step Cloning Kit*

Transfection of adenovirus Plasmids into 293A Packaging Cells

a. 293A cell culture should be prepared at least a day ahead to reach a confluence of 50%-70% monolayer morphology prior to transfection.

b. On the day of transfection, DMEM needs to be pre-warmed at 37℃ water bath and LipoGene™ transfection reagent should be equilibrated to room temperature and tapped to mix before use.

c. To prepare viral plasmids for each reaction using a 60-mm dish:

Table 1. Adenovirus vector system plasmids and transfection reagent required for transfection.

Component	Amount
pAd expression vector with GOI	2 μg
adenovirus genome backbone	4 μg
LipoGene™	30 μl

d. Mix plasmids with transfection reagent in DMEM and add drop-wise to pre-seeded 293A 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.*

Plaque Formation and Cell Collection

Plaque is an area of monolayer cells that display a cytopathic effect when infected by adenoviruses, usually observed as round, darker cells or white spots with microscope or naked eyes (Figure 1). It is important to observe viral plaques before collecting the transfected cells.

a. To minimize the spreading of adenovirus for a better condition of adenovirus plaque formation, low-melting-point agarose is suggested to be added in regular medium with a final concentration of 1.25%.
b. Small plaques can be visualized under microscope 10 to 21 days post-transfection. If the engineering sequence in the adenovirus expression plasmid carries fluorescent tags (GFP or RFP), the transfection efficiency can be estimated with fluorescence microscopy before production of plaques.
c. Pick an isolated viral plaque together with surrounding agarose, and transfer into 1 ml fresh medium and incubate overnight. In general, three to six plaques should be picked to compare their adenovirus titer, then the one with highest titer will be proceeded into subsequent experiments.

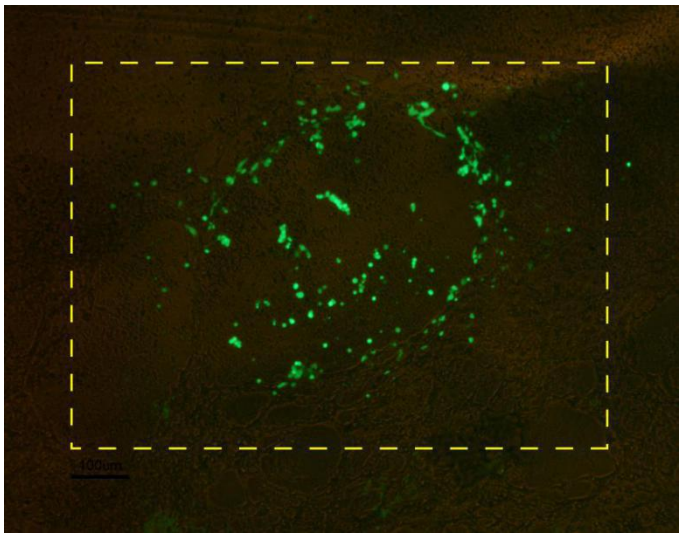

Figure 1. Identification of a plaque.

Note:

A stock solution of high-purity low-melting-point agarose can be prepared in sterile PBS to a final concentration of 5%. Before use, melt the stock completely in boiling water bath, and gradually cool down to 45 ℃ at room temperature. Dilute the agarose stock solution using pre-warmed 37 ℃ complete growth media to a final concentration of 1.25%. Immediately and gently add the well-mixed solution to cells with culturing medium removed ahead, and rotate to evenly covering the plaque cells. For a 6-well plate, add 3 ml agarose/medium per well.

It is important to observe viral plaques before collecting the transfected cells. To minimize the spreading of adenovirus for a better condition of adenovirus plaque formation, low-melting-point agarose is suggested to be added in regular medium, and small plaques can be visualized under microscope 10 to 21 days post-transfection. If the engineering sequence in the adenovirus expression plasmids (adenovirus shuttle vectors) carries fluorescent tags (GFP or RFP), the transfection efficiency can be estimated with fluorescence microscopy before production of plaques.

adenovirus Amplification

a. On the next day, add adenovirus-containing supernatant into fresh, pre-seeded 293A cells to amplify adenovirus.

b. Collect cells and supernatant when observing formation of plaques, and proceed into a freeze-thaw cycle 3 times before collecting all adenoviruses.

c. The collected adenovirus is recognized as passage 1 (P1 adenovirus). Then, infect fresh 293A cells with P1 adenovirus.

d. Perform infection-collection cycle for three times till P4 adenovirus is obtained, and expand adenovirus production into large-scale through P4 adenovirus infection. When formation of plaques is observed, adenoviruses are collected for purification and concentration.

Note:

1. *Use a cell scraper instead of trypsinization to detach cells. Collected cells should be centrifuged at 500 g, 4 ℃ for 10 min. Discard the most supernatant and leave 2ml to resuspend the cell pellet, transfer to a container at lower than -80 ℃ (using dry ice or liquid nitrogen) for freezing and thawing.*

2. *Immediately remove from the 37 ℃ bath when the adenovirus suspension melts completely in case of any decrease of the adenovirus titer. Shake the completely melted suspension heavily for 30 seconds. Usually, two to four rounds of freeze-thaw cycle can improve the yield of adenoviruses with high titer.*

3. *After freeze-thaw cycles, adenovirus lysate can be centrifuged at 500 g, 4 ℃ for 10 min to remove cell debris, and stored at -80 ℃ for later use.*

adenovirus Purification

The purification process of recombinant adenovirus is composed of three steps: PEG8000 condensation, CsCl density gradient centrifugation and dialysis. The detailed operation process is as following:

a. Thaw: Take the adenovirus out from -80 centigrade one day in advance, and melt in water bath at room temperature. Centrifuge at 7000 g, 4℃ for 10 min and collect supernatant.

b. PEG8000 condensation: Add 50 ml PEG8000 solution (20% PEG8000 in ultra-pure water with 2.5 M NaCl) per 100ml supernatant, placing on ice for 1 hour to pull-down adenoviruses (Time for incubation on ice can be relatively extended). Centrifuge the mixture for 20 min at 7000 g, 4℃, discard the supernatant and resuspend adenovirus pellet in 10 ml CsCl solution at density of 1.10g/ml (solvent of CsCl is 20mM Tris-HCl, pH8.0, please see the following CsCl solution preparation method in table 2). The adenovirus-containing CsCl solution should be pink (Figure 2).

Table 2. CsCl solution preparation.

Density at 20℃ (g/ml)	Concentration (ml/ml)	Amount of CsCl (g)	Final Volume (ml)
1.40	548.3	5.483	10
1.30	402.4	4.024	10
1.20	143.8	1.438	10

c. CsCl density gradient centrifugation: Place 2 ml of 1.40 g/ml CsCl solution on the bottom of a centrifuge tube, next add 3 ml of 1.30 g/ml CsCl solution slowly on top of the first layer, then add 5 ml adenovirus suspension. Centrifuge with Beckman SW28 rotor at 26,000 rpm, 4℃ for 2 hours.

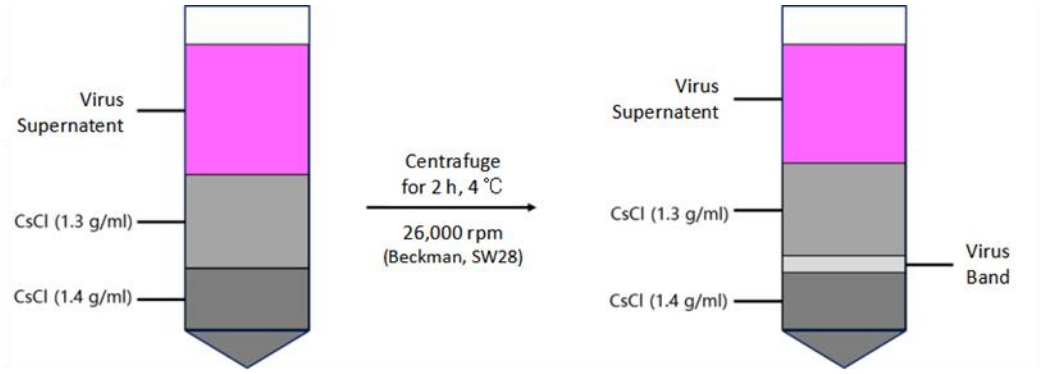

Figure 2. CsCl density gradient centrifugation process.
Left panel: CsCl gradient before ultracentrifugation.
Right panel: CsCl gradient after ultracentrifugation.

d. adenovirus collection: Collect adenovirus band between 1.30g/ml and 1.40g/ml layers with a syringe, transfer into dialysis bag.

Note:

The dialysis bag should be boiled in 10 mM EDTA-Na_2 for 10 min, cool down to room temperature before use.

e. Dialysis: Put the dialysis bag containing adenovirus in dialysis buffer (50 g sucrose, 10 ml 1 M Tris-HCl, PH 8.0, 2 ml 1 M $MgCl_2$, top up to 1 L by distilled water), and stir at 4℃ overnight. Replace with fresh buffer once during dialysis.

f. Formulation: Collect adenovirus from the bag, adjust volume to 500 µl with PBS, and determine the titer. Purified recombinant adenovirus should be kept in 4℃ for no more than a week or in -80℃ for long time storage.

Titration of Purified recombinant adenovirus

The titer determination method of recombinant adenovirus is plaque assay. Plaque-forming unit (PFU) is the number of plaques induced by certain volume of adenoviruses, representing the concentration of active viral particles.

Plaque assay of recombinant adenovirus:

a. Plate 293A cells in 60-mm dishes at least one day in advance.

b. When cell confluence reaches approx. 100%, add diluted adenovirus at different concentrations and incubated at 37℃.

c. 4 to 8 hours post infection, cover cells with 8 ml low-melting-point agarose solution (10% FBS, 1.25% agarose).

d. Calculate the titer of recombinant adenovirus by counting number of plaques in 9-11 days of culturing.

Note:

Titer of recombinant adenovirus can also be determined by observing fluorescence when applicable, or through the method of Western blotting (WB), immunofluorescence (IF) and immunohistochemistry (IHC) detection on expression level of target genes.

Transduction of Target Cells

For the reason that MOI varies in different cell lines, preliminary experiment is necessary to ensure a proper MOI of target cells before conducting formal experiments.

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 adenoviruses in different labs.

Cell Preparation

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

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 recombinant adenovirus

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

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

Day 1: Prepare adenovirus in a six-MOI gradient, and dilute proper amount of adenovirus 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 adenoviruses to pre-seeded cells and incubate for 4 to 8 hours at 37 ℃, then refresh the medium to remove adenoviruses.

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

Note:

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

Transduction

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

a. For adherent cells:

Recombinant adenovirus containing target gene and the same amount of control adenovirus should be added separately into two groups of cells and mixed well. The amounts of adenoviruses to be used are based on size of container described in the following table. For MOI test in most cell types, a gradient of 3, 10, 30, 100, 300 to1000 at three replicates would be sufficient enough. Refresh medium in 4 to 8 hours. Protein of interests can be detected within 48-72 hours with fluorescence microscopy, WB, etc.

Table 3. adenoviruses amounts in different container size.

Size of Container	Surface Area (cm^2)	Volume of Medium	Volume of
96-well	0.3	100 µl	0.1-0.5 µl
24-well	2	500 µl	1-3 µl
12-well	4	1 ml	2-5 µl
6-well	10	2 ml	5-20 µl

For example: If the tier of recombinant adenovirus is 5 × 10^{11} PFU/ml, dilute to 5 × 10^{10} PFU/ml (10-fold) with complete growth medium of target cells. When there are 1 × 10^5 cells in one well, and the MOI is 1,000, required volume of diluted adenovirus (5 × 10^{10} PFU/ml) should be (cell number) × (MOI) ÷ (PFU/ml of recombinant adenovirus) = 1 × 10^5 × 1,000 / 5 × 10^{10} (ml) = 2 µl. Thus, 2 ul of diluted adenovirus should be added into this well.

Note:

The waste should be disposed following procedures described in Biosafety Requirements Section.

b. For suspension cells:

Spin infection is a sufficient way to transduce suspension or semi-suspension cells. In brief, seal the cell culture plate by parafilm after adding adenoviruses, spin in a low-speed swinging-bucket centrifuge at 200g for 1 hour at 37℃,

and culture cells at 37℃ overnight. Medium should be refreshed the next day.

If the condition is not allowed for spin infection, a centrifuge tube can be used instead by transferring cells into a tube and centrifuge at low-speed. Discard most of the supernatant after centrifugation, add adenoviruses, and incubate at room temperature for 15-30 min. Then transfer the cell-adenovirus mixture into a proper container, and culture at 37℃ overnight. Medium should be replaced the next day.

Determine Transduction Efficiency

48 to 72 hours post-transduction, fluorescent proteins can be observed when applicable, and the alteration of target gene can be analyzed at mRNA-level by qPCR or at protein-level by Western blot (WB).

Safe Use of adenovirus (AdV)

1. Adenovirus 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 adenovirus suspension. If biosafety cabinet is contaminated with adenovirus during operation, scrub the table-board with solution comprising 70% alcohol and 1% SDS immediately. All tips, tubes, culture plates, medium contacting adenovirus 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. Adenovirus related animal experiments should also be conducted in BL-2 level.

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

7. Wash hands with sanitizer after experiment.

Storage and Dilution of Adenovirus

Storage of Adenovirus

Adenovirus can be stored at 4°C for a short time (less than a week) before using after reception. Since adenoviruses 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 adenovirus titer redetection is suggested before using if the adenoviruses have been stored for more than 12 months.

Note: 1. Repeated freeze-thaw cycles must be avoided in case of a downside effect on adenovirus titer (for each freeze-thaw cycle, there would be a 10%-50% decrease). Genemedi will provide recombinant adenovirus products in small aliquots (200 μl/tube) that can be directly stored in -80 centigrade for multiple usage.
2. For adenoviruses stored for more than 6 months, it is suggestive to re-analyze adenovirus titer before use.

Dilution of Adenovirus

To properly thaw recombinant adenovirus frozen aliquots, transfer adenoviruses from -80℃ freezer to an ice-water bath till completely melted. When melted, add proper amount of sterile PBS or serum-free culture medium, and keep in 4℃ for no more than a week.

Precautions

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

- Avoid introducing air into the Adenovirus samples during vortexing, 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 adenoviruses 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 Adenovirus 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 Adenovirus into low salt solution. Some adenoviruses aggregate in low salt solution, which will be non-infectious.

References

1. Luo J. et al. A protocol for rapid generation of recombinant adenoviruses using AdEasy system. Nat. Protocols. 2 (5), 1236-1247 (2007).
2. He T. C. et al. A simplified system for generating recombinant adenoviruses. PNAS. 95, 2509-2514 (1998).
3. Meier O. & Greber U. F. adenovirus endocytosis. J. Gene Med. 6 (Suppl 1), S152-S163 (2004).

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AAV2 variant (Y444F,Y730F,Y500F,Y272F,Y704F,Y252F) vector system			AAV8-3m vector system	AAV9-PHP.eB vector system
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GM-T00348	ABL T315I	25	ABL1	ABL, CHDSKM, JTK7, bcr/abl, c-ABL, c-ABL1, p150, v-abl	Introcelluar Protein		Inquiry	Detail
GM-T97036	C-peptide	197	AHSG	A2HS, AHS, APMR1, FETUA, HSGA	Secreted Protein/Potential Cytokines	Therapeutics Target, Diagnostics Biomarker	Inquiry	Detail
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GM-T55654	CFTR	1080	CFTR	ABC35, ABCC7, CF/MRP, MRP7, TNR-CFTR, dJ760C5.1, CFTR	Transmembrane Protein	Therapeutics Target	Inquiry	Detail
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GM-T01110	CSF-1R	1436	CSF1R	C-FMS, CD115, CSF-1R, CSFR, FIM2, FMS, HDLS, M-CSF-R	Transmembrane Protein		Inquiry	Detail

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