Production protocol

Adenovirus (AdV)

Introduction of Recombinant Adenovirus (rAdV)

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 rAdV 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 rAdV. Moreover, rAdV is easy to operate and expand into large-scale, and the efficiency can reach up to 100%. Thus, rAdV 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 virus particles further migrate into cell nucleus for self-replication using replication machinery of the host [3]. Once replicated, the virus genome is assembled into its protein shell and released from cells, causing cell lysis [3].

Nowadays, several packaging systems of rAdV are developed, in which AdEasy1 and AdMAX2 are the two most popular ones, sharing a common strategy that target gene sequence is cloned into a 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 virus replication1. Thus, packaging of rAdV 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 virus titer during virus production. This rAdV protocol is developed according to AdMAX system, using a two-vector system composed of a pAd shuttle vector and a rAdV backbone vector pBHGlox(delta) E1-3cre.

Protocol Overview

A schematic overview of recombinant 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 rAdV shuttle vectors. The inverted terminal repeat (ITR) sequences present in these vectors provide all of the cis-acting elements necessary for rAdV replication and packaging.

The recombinant expression plasmid is co-transfected into the 293A cells (an E1-complementing cell line) with packaging plasmid pAd-BHGlox(delta)E1, E3, which together supply all of the trans-acting factors required for AdV 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 virus titer, then select the one with highest titer to proceeded subsequent amplification, concentration and purification experiments.

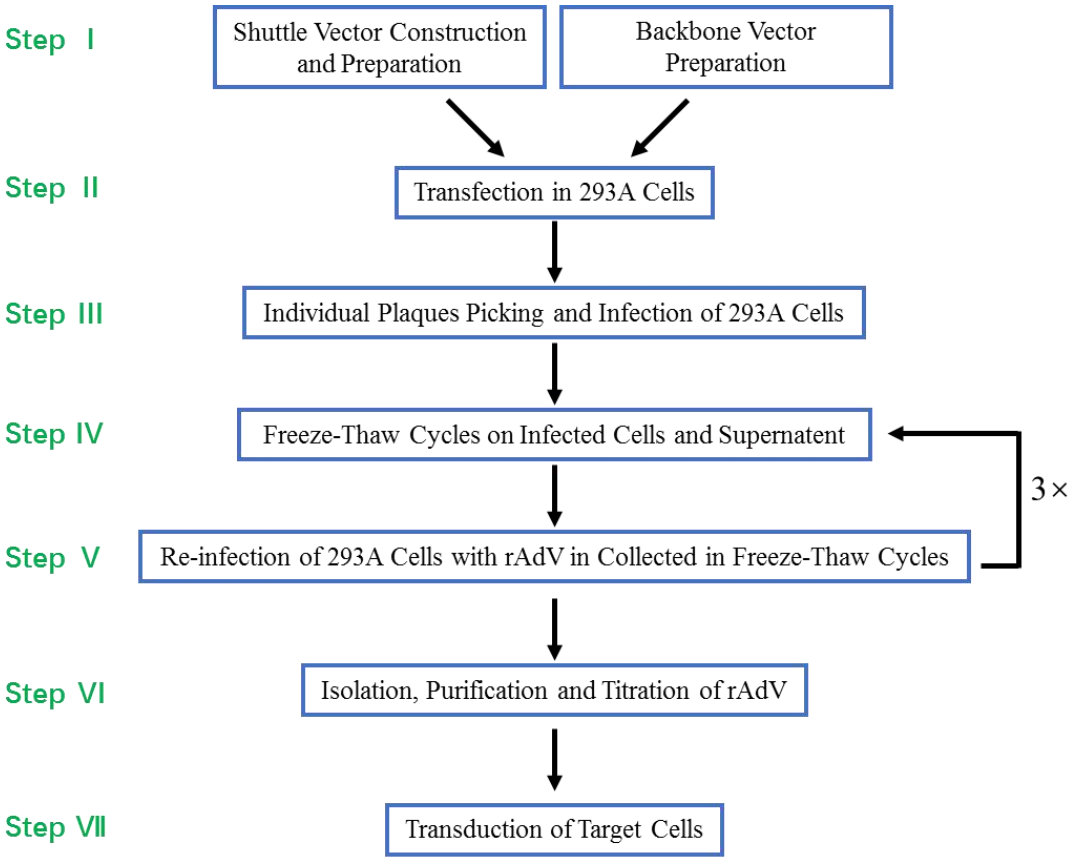

Figure 1. AdV packaging experiment flow chart.

Experimental Materials

Virus Packaging System

A two-plasmid system is used for packaging recombinant adenovirus (rAdV) in this protocol, which includes a shuttle vector (pAd) that can be cloned into engineering sequences for gene overexpression, RNA interference and CRISPR/Cas9 gene knockouts, and an adenoviral backbone vector (pBHGlox(delta) E1, 3Cre). For more information regarding how to choose the right shuttle vector for different experimental purpose, please consult our AdV User Manual.

Bacterium Strain

E. coli strain DH5α is used for amplification of shuttle and backbone vectors.

Packaging Cell Line

293A is the virus packaging cell line that can facilitate initial production, amplification and titer determination of rAdV. 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 AdV

Vector Construction of AdV

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

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）.

Transfection of Virus 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 in 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. Plasmid and transfection reagent required for transfection.

Component	Amount
pAd shuttle plasmid	2 μg
pGlox(delta)E1, 3Cre	4 μg
LipoGene™	30 μl

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

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 virus for a better condition of virus 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 shuttle 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 virus titer, then the one with highest titer will be proceeded into subsequent experiments.

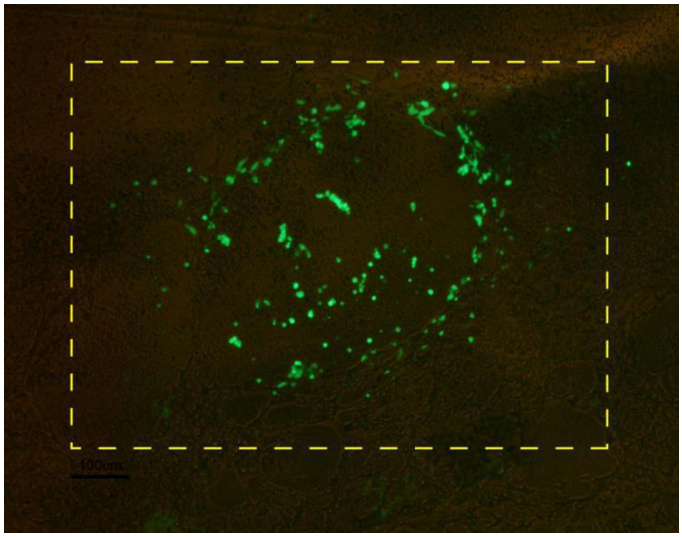

Figure 1. Identification of a plaque.

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 ℃ in 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 virus for a better condition of virus 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 shuttle plasmid carries fluorescent tags (GFP or RFP), the transfection efficiency can be estimated with fluorescence microscopy before production of plaques.

Virus Amplification

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

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

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

d. Perform infection-collection cycle for three times till P4 virus is obtained, and expand virus production into large-scale through P4 virus infection. When formation of plaques is observed, viruses 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 virus suspension melts completely in case of any decrease of the virus titer. Shake the completely melted suspension heavily for 30 seconds. Usually, two to four rounds of freeze-thaw cycle can improve the yield of viruses with high titer.*

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

Virus Purification

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

a. Thaw: Take the virus 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 viruses (Time for incubation on ice can be relatively extended). Centrifuge the mixture for 20 min at 7000 g, 4℃, discard the supernatant and resuspend virus 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 virus-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 virus suspension. Centrifuge with Beckman SW28 rotor at 26,000 rpm, 4℃ for 2 hours.

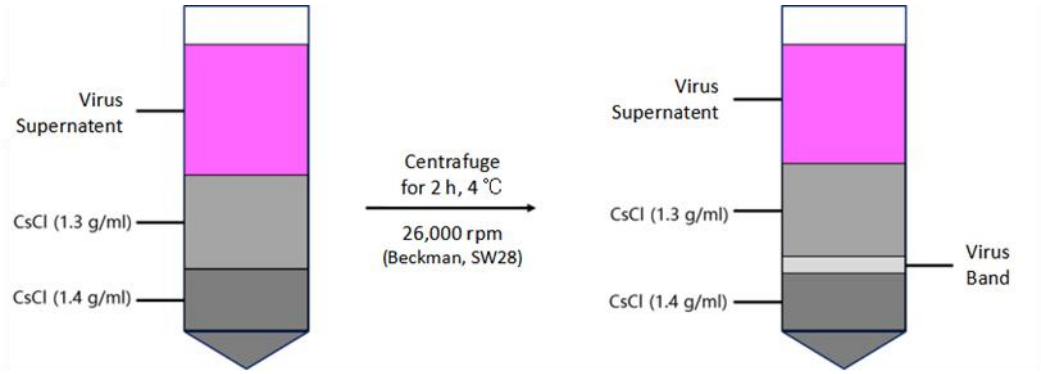

Figure 2. CsCl density gradient centrifugation process.
Left panel: CsCl gradient before ultracentrifugation.
Right panel: CsCl gradient after ultracentrifugation.

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

Titration of Purified rAdV

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

Plaque assay of rAdV:

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

b. When cell confluence reaches approx. 100%, add diluted virus 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 rAdV by counting number of plaques in 9-11 days of culturing.

Note:

Titer of rAdV 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 viruses 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 rAdV

Prepare the virus 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 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.

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.

Transduction

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

a. For adherent cells:

rAdVs containing target gene and same amount of control viruses should be added separately into two groups of cells and mixed well. The amounts of viruses 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. Viruses amounts in different container size.

Size of Container	Surface Area (cm^2)	Volume of Medium	Volume of Viruses
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 rAdV 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 virus (5×10^{10} PFU/ml) should be (cell number) × (MOI) ÷ (PFU/ml of rAdV) = $1 \times 10^5 \times 1{,}000 / 5 \times 10^{10}$ (ml) = 2 µl. Thus, 2 ul of diluted virus 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 viruses, 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 viruses, and incubate at room temperature for 15-30 min. Then transfer the cell-virus 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. AdV 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. AdV related animal experiments should also be conducted in BL-2 level.

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

7. Wash hands with sanitizer after experiment.

Storage and Dilution of AdV

Storage of AdV

Virus can be stored at 4°C for a short time (less than a week) before using after reception. Since AdV viruses 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 AdV viruses have been stored for more than 12 months.

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

Dilution of AdV

To properly thaw rAdV frozen aliquots, transfer viruses 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 AdV exposure to environmental extremes (pH, chelating agents like EDTA, temperature, organic solvents,

protein denaturants, strong detergents, etc.)

• Avoid introducing air into the AdV 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 AdV 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 AdV 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 AdV into low salt solution. Some viruses aggregate in low salt solution, which will be non-infectious.

References

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).

Contact Information

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