13/11/1390
One Day

Workshop

Gene Delivery By Lentiviral Vector

Autoimmune Diseases Research Center
Shiraz University of Medical Sciences

PREFACE:

Gene delivery is the process of introducing foreign DNA into host cells. Gene delivery is, for example, one of the steps necessary for gene therapy and the genetic modification. There are many different methods of gene delivery developed for a various types of cells and tissues, from bacterial to mammalian. Generally, the methods can be divided into two categories, non-viral and viral.

Non-viral methods include physical methods such as microinjection, gene gun, impalefection, hydrostatic pressure, electroporation, continuous infusion, sonication and chemical, such as lipofection. It can also include the use of polymeric gene carriers (polyplexes).

Virus mediated gene delivery utilizes the ability of a virus to inject its DNA inside a host cell. A gene that is intended for delivery is packaged into a replication-deficient viral particle. Viruses used to date include retrovirus, adenovirus, adeno-associated virus and herpes simplex virus.

Introduction:

Viruses are logical tools for gene delivery. They replicate inside cells and therefore have evolved mechanisms to enter the cells and use the cellular machinery to express their genes. The concept of virus-based gene delivery is to engineer the virus so that it can express the gene of interest and yet retain its ability to deliver the gene to target cells. These engineered viruses are often referred to as viral vectors. Depending on the specific application and the type of virus, most viral vectors contain mutations that hamper their ability to replicate freely as wild-type viruses in the host.

Viruses from several different families have been modified to generate viral vectors for gene delivery. These viruses include adenoviruses, adeno-associated viruses, herpes simplex viruses, picornaviruses, alphaviruses, and retroviruses.

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Retroviruses were the first viruses to be modified for gene delivery, and retroviral vectors are used in the majority of all gene therapy clinical trials (Anderson, 1996).

During the past decade, gene delivery vehicles based on human immunodeficiency virus type 1(HIV-1), the best characterized of the lentiviruses, have been developed. Lentiviral vectors derived from HIV-1 are capable of transducing a wide variety of dividing and nondividing cells, integrate stably into the host genome, and result in long-term expression of the transgene (siRNA, cDNA, DNA fragments, antisense, and ribozymes). The HIV-1genome contains nine open reading frames (ORFs) encoding at least 15 distinct proteins involved in the infectious cycle, including structural and regulatory proteins. In addition, there are a number of *cis*-acting elements required at various stages of the viral life cycle. The general strategy used to produce vector particles has been to eliminate all dispensable genes from the HIV-1 genome and separate the *cis*-acting sequences from those *trans*-acting factors that are absolutely required for viral particle production, infection, and integration.

The widely used third generation of lentiviral vectors consists of four plasmids. The transfer vector contains the transgene to be delivered in a lentiviral backbone containing all of the *cis*-acting sequences required for genomic RNA production and packaging. The transfer vector also contains the woodchuck hepatitis virus regulatory element (WPRE) that enhances expression of the transgene and a central polypurine tract (cPPT) purported to increase efficiency of nuclear import of the preintegration complex. The packaging system involves three additional plasmids that provide the required *trans*-acting factors.

Viral particles can be pseudotyped with a variety of envelope proteins. One commonly used envelope protein is the vesicular stomatitis virus protein G (VSV-G), which is incorporated into the viral membrane and confers the ability to transduce a broad range of cell types, including primary cells, stem cells, and early embryos. In addition, an important safety feature is provided by a deletion in the 3′ LTR (long terminal repeat) that results in replicationdefective particles. During reverse transcription, the proviral 5′ LTR is copied from the 3′ LTR, thus transferring the deletion to the 5′ LTR. The deleted 5′ LTR is transcriptionally inactive, preventing viral genomic RNA production from the integrated provirus. When these four plasmids are transfected into 293T human embryonic kidney cells, viral particles accumulate in the supernatant.

Safety consideration:

Lentiviral vectors are classed as biohazard risk group 2 and must be handled in a containment level 2 laboratory (CL2) using containment level 3 operational practices. The following containment level 3 operational practices must be followed:

1. Exterior street clothing such as coats, boots and gloves and all jewelry including watches must be removed before personnel enter the containment laboratory. Dedicated laboratory clothing such as a lab coat must be donned upon entering the laboratory and must be removed before leaving. Lab coats must be stored in the lab.
2. All personal items such as purses, bags, sweaters, coats and boots must not be brought inside the containment laboratory and therefore must be stored outside the lab in lockers or offices.
3. Two pairs of gloves must be worn when manipulating the vector. Always treat your outer gloves as contaminated.
4. A solid front disposable gown must be worn over laboratory clothing when manipulating the vector. If a spill or splash occurs, autoclave and dispose of gown.
5. Limit the use of hypodermic syringes and needles and other sharps. Strongly consider the use of engineered sharps systems. If feasible, use plastic disposable transfer pipettes rather than glass Pasteur pipettes.

6. All manipulations of the vector must be conducted in a Biological Safety Cabinet (BSC). Only one person at a time working in the BSC.

7. Work in the BSC over an absorbent pad.

8. A small stand and autoclave bag must be placed in the BSC to collect waste (including disposable pipettes). Immediately upon completing work, seal it in a second autoclave bag and bring it to the central autoclave facility.

9. A tray containing freshly diluted **10% bleach solution** must be placed in the BSC to collect reusable glass pipettes. Pipettes must be submerged for a minimum of 30 minutes.

10. A yellow sharps container must be placed inside the BSC for sharps disposal.

11. Centrifugation of infectious materials must be carried out in closed containers placed in sealed safety cups or rotors that are uploaded and unloaded in a BSC.

12. Inventory and storage locations of viral samples must be tracked.

13. Containment laboratory doors must be kept closed at all times and must be locked when unoccupied.

14. Lentiviral vectors must be stored inside the containment laboratory.

Modes of Transmission

A) High Risk Exposures:

i. Skin puncture or injection

ii. Ingestion

iii. Contact with mucous membranes (eyes, nose, mouth)

iv. Contact with non-intact skin

B) Low Risk/Potential Exposure:

i. Bite from a recently infected animal

ii. Percutaneous contact with body fluids from a recently infected animal

iii. Aerosols

First Aid

- Should a skin exposure occur, immediately go to the sink and thoroughly wash the area with soap and water.

- For a skin wound, immediately go to the sink and thoroughly wash the wound with soap and warm running water for 15 minutes. Gently work the blood toward the wound and pat dry.

- A splash to eye(s), nose or mouth requires immediately flush of the area with running water for at least 10 minutes.

- A splash affecting garments requires removal of the garments that have become soiled or contaminated, place in an autoclave bag and decontaminate before washing.

Materials and Methods:

Transformation of bacterial cells

Materials:

- Ice
- Chilled 14 mL (round-bottomed polypropylene bacterial culture) centrifuge tube
    - 1 per transformation
    - 1 for control DNA
- Chilled (4^{o}C) pipette tips (yellow + white)
- 42^{o}C exactly waterbath
- Timer
- Chilled (4^{o}C) SOC medium (900 µL each transformation)
- 37^{o}C shaking incubator @ 225 rpm
- 1.5% LB agar + Kanamycin (50 µg/mL; CP/control pep DNA constructs) or Ampicillin (100 µg/mL; control DNA in kit or lenti packaging plasmids from Grant)
- 1 µL pipette
- 10 µL pipette

Preparation:

- Melt agar in microwave.
- Add appropriate antibiotic into 100 mL petri dishes.
    - 10 x 500 µL aliquots of each antibiotic stored at -20^{o}C
        - Filter sterilised
        - Never reuse
        - Thaw on ice before use (~30 min)
    - Kanamycin stock:

        - 10 mg/mL (added 100 µL per plate)
    - Ampicillin stock:
        - 50 mg/mL (added 40 µL per plate)
- Pour 20 mL of agar into each plate when agar is just cool enough to touch.
- After agar sets (~20 min) place plates at 4^{o}C overnight before use. (Plates can be stored at 4^{o}C for 7 – 10 days, before antibiotic goes off)

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Method:

1. Chill the 14 mL centrifuge tubes.
2. Remove frozen Competent Cells from -70^{o}C, and place on ice for 5 min or just until thawed.
3. Gently mix the thawed Competent Cells by flicking the tube.
4. Transfer 100 µL of Competent Cells into each chilled tube. Note: Pipette quickly or use chilled (4^{o}C) pipette tips to prevent cells from warming above 4^{o}C.
5. Add 1 – 50 ng DNA (in 10µL volume) to each tube.
6. Add 1 µL (0.1ng) of Competent Cells Control DNA to a tube. [Control, to determine the transformation efficiency]
7. Immediately place tubes on ice for 10 min.
8. Heat shock the cells for 45 – 50 sec in a water bath at exactly 42^{o}C. Do not shake.
9. Immediately place tubes on ice for 2 min.
10. Add 900 µL of cold (4^{o}C) SOC medium to each tube.
11. Incubate at 37^{o}C for 60 min with shaking @ 225 rpm.
12. Dilute each reaction 1:10 and 1:100. (For [Control] dilute the cells 1:10. Plate 100 µL on LB/ampicillin plates)
13. Plate 100 µL undiluted cells, and 1:10 and 1:100 dilutions on antibiotic plates.
14. Incubate plates at 37^{o}C for 12 – 14 hrs.
15. For [Control] dilute the cells 1:10. Plate 100 µL on LB/ampicillin plates.
16. Equation for Transformation Efficiency (cfu/µg):

$$\frac{cfu\ on\ control\ plate}{ng\ of\ Competent\ Cells\ Control\ DNA\ plated}\ x\ \frac{1\ x\ 10^{3}ng}{\mu g} = \_\_\_\_\_\_ cfu/\mu g$$

17. Pick up colonies which grown on antibiotic plates and subculture in new antibiotic plates.

18. Perform a Restriction Digestion reaction to confirm your cloning of desire plasmids.
19. After confirmation, prepare stocks of transformed bacteria with desire plasmids.
20. Perform plasmid purification using Maxiprep kit.

Lentivirus Production

Calcium Phosphate Transfection:

Materials:

- HEPES- buffered Saline (HBS) 2x (pH 7.05, filter sterilized)
    - 0.28M NaCl
    - 50mM HEPES
    - 10mM KCl
    - 1.5mM Na_2HPO_4
- 2M $CaCl_2$

Protocol:

1- Plate 4-5 x 10^6 HEK293.T in T75 flask in DMEM containing 10% FBS and incubate 24 hours before transfection.

2- Replace the medium by fresh medium 2h before the transfection.

3- Dilute 4 lentiviral plasmids at 3:1:1:1 ratio (40µg FC) in 650µl ddH_2O and mix carefully.

4- Add 93µl 2M $CaCl_2$ (do not mix).

5- Drop wise add 750µl 2X HBS and mix gently by swirling.

6- Incubate the mixture at room temperature for 30min.

7- Add the complex drop by drop to the cells and gently rocking the flask side to side.

8- Incubate the cells at 37 ^{0}C in 5% CO_2.

9- Check your transformation after 48-72 hr by using Felowcytometry or Florescent microscopy.

Virus collection:

1- Day 2 at 8am (24h after transfection) and store at 4 ^{0}C. Replace the medium.

2- Day 2 at 8pm. Collect the supernatant and store at 4 ^{0}C.

3- Day 3 at 8am (repeat the step 2).

4- Day 3 at 8pm (repeat the step2).

5- Day 4 at 8am (repeat the step2).

6- Pool all virus supernatants harvested and filter with 0.25µM filter.

7- Distribute in small aliquots and store at -70 ^{0}C.

Transduction Protocol:

1- Plate the mammalian cell line of choice in complete medium 24h prior to transduction (50-70% confluent).

2- Thaw the Lentiviral stock slowly. Add Polyberen to each tube (6-10µg/ml depending of cell type).

3- Remove the viral particle-containing medium and replace it with fresh medium.

4- For stable expression experiments remove the medium and replace it with fresh complete medium that contains the appropriate amount of antibiotics for selection of transduced cells.

5- Replace medium with fresh, antibiotic-containing medium every 3-4 days until resistant colonies can be identified.

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Checking viral production:

Method:

a) One medium size flask of HEK cells was trypsinized (2 ml of trypsin) and cells were counted (0.96 x10^6/ml). After that the following materials are added to a 6 well culture dish.

1) 10 µg/ml of polyberene 2) No virus 3) 2.5 ml media 4) 2.5x10^5 HEK cells in 0.5 ml	1) 10 µg/ml of polyberene 2) 100 µl of concentrated media containing virus 3) 2.4 media 4) 2.5x10^5 HEK cells in 0.5 ml	1) 10 µg/ml of polyberene 2) 50 µl of concentrated media containing virus 3) 2.45 media 4) 2.5x10^5 HEK cells in 0.5 ml
1) 10 µg/ml of polyberene 2) 2.5 ml of 1/5 diluted of unconcentrated media virus 3) 2.5x10^5 HEK cells in 0.5 ml		

b) After addition of different materials, plate was moved horizontaly a few times and put in incubator about 60-72 hrs.

c) After incubation, media is removed carefully and cells washed very carefully with PBS and trypsinized and transfer to a small (T25) flask for growing and after that passaging to check by flow cytometer and/or florescent microscope and also collecting the supernatant (if the cell would be positive by flowcytometer).

All the best.