Video Article

Lentivirus Production

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Abstract

RNA interference (RNAi) is a system of gene silencing in living cells. In RNAi, genes homologous in sequence to short interfering RNAs (siRNA) are silenced at the post-transcriptional state. Short hairpin RNAs, precursors to siRNA, can be expressed using lentivirus, allowing for RNAi in a variety of cell types. Lentiviruses, such as the Human Immunodeficiency Virus, are capable to infecting both dividing and non-dividing cells. We will describe a procedure which to package lentiviruses. Packaging refers to the preparation of competent virus from DNA vectors. Lentiviral vector production systems are based on a 'split' system, where the natural viral genome has been split into individual helper plasmid constructs. This splitting of the different viral elements into four separate vectors diminishes the risk of creating a replication-capable virus by adventitious recombination of the lentiviral genome. Here, a vector containing the shRNA of interest and three packaging vectors (p-VSVG, pRSV, pMDL) are transiently transfected into human 293 cells. After at least a 48-hour incubation period, the virus containing supernatant is harvested and concentrated. Finally, virus titer is determined by reporter (fluorescent) expression with a flow cytometer.

Video Link

The video component of this article can be found at https://www.jove.com/video/1499/

Protocol

Part 1: Transfection of HEK 293 Cells To Produce Lentiviruses

*24 hours before transfection, plate 2.5 X 10^6 cells in a 10cm dish for a confluency of 50-70% the next day.

1. Start this protocol by preparing the DNA with which one will later transfect one's cells to make virus. First, dilute FuGENE 6 Transfection Reagent Roche, a lipid reagent that make cells take up DNA. In a 1.5 ml tube, pipette 30µl FuGENE 6 into 600ul serum-free medium (SFDMEM). Be careful to not let FuGENE touch the side of the tube as it is delivered into the medium. Let this FuGENE and SFDMEM mix and incubate at room temperature for 5 minutes.
2. In the cap of the tube, mix 4 µg lentiviral plasmid with 4 µg each 3rd generation viral packaging vectors (pMDL, pRSV and pVSV-G). The lentiviral plasmid contains the DNA the virus will insert into the genome of every cell it infects, while the packaging vectors contain genes for all the other proteins required to make a lentivirus.
3. Close the lid and mix by inverting the tube a few times.
4. Incubate 15-20 minutes at room temperature.
5. Pipette entire content of the tube into 293 plate. Mix gently by tilting plate back and forth.
6. Return the cells to the incubator and allow viral production to continue for 48-96 hours before harvest.
7. During incubation, the 293 cells transcribe the DNA of interest from the lentiviral plasmid, creating an RNA copy of the lentiviral construct. They also transcribe and translate the genes in the packaging vectors into viral proteins. These proteins proceed to make viruses containing the RNA version of your lentiviral construct, which the virus will reverse transcribe and insert into the genomes of all the cells it infects.

Part 2: Lentiviral Harvest

1. After incubation period, take cells out of the incubator and use a disposable, 10ml syringe to remove the supernatant, which now contains the virus. There will be about 10ml of supernatant.
2. Attach a 0.45µM syringe filter to the tip and filter the virus into a Beckman Ultracentrifuge tube. The filter allows only viruses, and not cells to pass through.
3. Before discarding, incubate all virus-producing cells, culture plates, syringes and filters in 10% bleach for 45 minutes.

Part 3: Lentiviral Concentration via Ultracentrifugation

1. Load centrifuge tubes into SW-41Ti or SW-28 rotor buckets.
2. Use serum-free DMEM to balance all virus-containing tubes to within 0.2g.

3. Seal shut rotor buckets and hook them onto rotor before placing rotor inside the ultracentrifuge
4. Spin the virus for 90 minutes in an ultracentrifuge at 4°C at 25,000 rpm in an SW-41Ti rotor or 24,000rpm in an SW-28 rotor.
5. In a tissue culture hood, invert tubes upside down to pour supernatant into a container containing 10% bleach.
6. Use a Kimwipe to absorb excess liquid around the pellet and invert the tube in a convenient rack for no more than five minutes.
7. To re-suspend the viral pellet, use a filter tip to add 100 µL sterile PBS to the tube, then pipette up and down about 20 times.
8. Leave the virus at 4°C overnight to complete re-suspension. One can store viral preps at 4 degrees for about 1 week and at -80 degrees for up to one year. Remember to treat all empty tubes with 10% bleach before discarding.
9. If one intends to use the viral prep for longer than the 1 week, make minimal aliquots of 5-20µl for future experiments and 1 aliquot of 3 to 5µl for the titration. Freeze and store the aliquots at minus 80.

Part 4: Lentiviral Titration Using Flow Cytometry

1. The next step is to determine the viral titer by flow cytometry. This method only works in cases where the lentiviral plasmid contains a fluorescent reporter gene. The idea is that when the virus infects a cell, it introduces the lentiviral plasmid DNA into that cell's genome. The infected cell will then transcribe and translate the genes you included in the lentiviral plasmid, and if you included a fluorescent reporter gene, infected cells will fluoresce. The level of fluorescence represents the potency, or titer, of the virus.
2. For each viral stock to be titered, dilute 3 µl of concentrated virus into 1.5 ml of complete media (DMEM, pen-strep, glutamine, FBS).
3. In wells 1-6 in a single row of a 96 well plate, perform the following serial dilutions: to all 6 wells add 100 µl of complete DMEM media. To the first well add 100µl of media with no virus (this is your unstained control), to the second well add 100 µl of the diluted virus (which is equivalent to1 ml of undiluted virus), to the third well add 100 µl of the mix from the second well, to the fourth well add 100 ml from the 3rd well, to the fifth well add 100µl from the 4th well, and to the sixth well add 100 µl from the 5th well. Finally take 100ul out of the 6th well and discard in bleach. A serial dilution was performed where each well has half the volume of virus compared to the one before it.
4. Bring the total volume in all wells to 200µl. Note that these are only suggested dilutions, which should work well for titering most concentrated viruses. If the virus is low in titer, or unconcentrated one may need to adjust the dilutions.
5. Treat any unused diluted virus with 10% bleach for 45 minutes before discarding into a biohazard waste container.
6. Add ~15,000 healthy, actively dividing 293 cells to each of the 6 wells. A single 96-well plate can be used to titer 16 viral preps.
7. Return cells to 37 degree incubator with 5% CO_2 and allow infection to proceed for at least 48 hours.
8. After the incubation, the cells are analyzed for reporter expression (in our case fluorescence) with a flow cytometer. Viral particles are quantitated by the percent of fluorescent cells per well.

Calculate the number of infectious units per ml with the following formula:

$$\frac{\text{(\# of cells per well on day of infection X reporter positive cells) X 1000}}{\text{ml of vector added to well}}$$

Part 5: Representative Results:

After a 48-hour incubation period post transfection, the plate of 293 cells should be at around 90% fluorescent. A high percentage of fluorescent cells is an indication of a high percentage of virus producing cells.

After centrifugation, the viral pellet is barely visible when resuspending. This is normal as the pellet is clear and very small.

When interpreting flow cytometer results, note that one can only achieve accurate titer calculations when infected (fluorescent) cells have received no more than one viral integration per cell. In order to ensure that this is the case, use cells with < 15 % infection rate for calculations. Cells with higher infection rates have likely received multiple viral integrants per cell. The dilutions suggested in step 2 should yield several samples within this range of infection. Ideally, use several samples to generate a titration plot (linear line) from which you can calculate the final titer, but you can calculate titer using a single sample, if necessary. One can expect titer of 1.0 x 10^7 TU/ml for un-concentrated virus and 1.0 x 10^8 TU/ml for concentrated virus.

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