Amplification of Cre-expressing Adenovirus in 293T cells

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Background on AdenoCre IRES EGFP system

(Vector Biolabs, Ad-Cre-IRES-GFP, cat# 1710), titer is 1x10^{10} PFU/ml)

AdenoCre/EGFP expresses both Cre recombinase and enhanced green fluorescent protein (EGFP). The EGFP is in an IRES (internal ribosomal entry site) expression cassette, a viral sequence that allows translation initiation in the middle of a mRNA sequence as opposed to the usual mechanism of translation using the 5' cap of mRNA.

Cre Recombinase is a topoisomerase that catalyzes the site-specific recombination of DNA flanked by unique DNA sequences known as loxP sites. In this way, infection with AdenoCre virus can be used to acutely delete or activate a gene through recombination in cells containing LoxP sites.

It should be noted that viral amplification must be done in 293T cells, which specifically express the E1 gene required for Adenoviral amplification. (Labs use replication-deficient E1-deleted virus for safety purposes.) Other cell types can be infected by Adenovirus, but cannot actually amplify the virus because they do not contain the E1 gene.

Adenovirus can grow up to 10,000 copies per one 293T cell. Usually you will get ~10^7 cells in one confluent 100-mm dish so the estimated viral titer will be ~10^11 pfu / dish. It depends on the condition of 293T cells and also on the quality of the original viral stock. This is why it's important to use healthy 293T cells with low passage numbers.

A practical note: Adeno control IRES-EGFP (empty vector)-infected cells tend to express higher level of EGFP possibly because introducing an additional sequence upstream of IRES may result in inefficient ribosomal entry. Hence, usage of EGFP fluorescence for titration may not be ideal, although is the most straightforward.

Viral amplification in 293T cells

I. *Preparation of 293T cells. [Usage of 293T cells with low passage numbers (optimally less than 15) is critical to obtain high titer adenovirus]*
   1. Grow 293T cells in 6 10-cm plates in DMEM/10% FBS.
   2. Grow 3 plates to confluency and move to part II for first-round virus production.
   3. Use 3 plates to generate more 293T for second-round virus production (part III). To do this, trypsinize and collect cells, then replate into 15 10-cm dishes. Allow these to grow to confluency before moving on to part III.

II. *First round of infection:*

If you do not have a lot of virus in hand, I would suggest to infect 1x10cm dish cells in the first round, then to 3 and 15 dishes in 2nd and 3rd round infection.

4. For 3 confluent plates, remove media from each plate and replace with 5 mL fresh media (DMEM/10% FBS) plus **1 μl stock virus at 1x10^{10} PFU/ml) per 1 million cells**. Incubate cells with virus overnight @ 37C/5% CO2.
   → Adjust your starting volume of virus based on its concentration in PFU/ml. Infection should be 10 PFU per cell. So, if you have a plate with 5 million cells, you want 50 million PFU. Thus you would start with 5 μl of virus at 1x10^7 PFU/μL or 1x10^10 PFU/ml. If you have 10 million cells, infect with 10 μl, etc. A typical confluent 10cm dish of 293T will be 5-10 million cells.
5. After overnight incubation, add 4 mL DMEM/10% FBS to each plate. Let cells grow for ~4 days, until GFP expression is seen in 100% of cells; the cells should be rounded up and a third or half of cells are detached already.
6. To collect virus, pipette gently to float all the cells (do not use trypsin) and transfer cells/media to falcon tubes.
7. Spin cells down for 5 minutes (setting 1), remove supernatant and resuspend in 1 mL PBS.
8. To lyse cells, freeze cells in a dry ice/methanol bath, then thaw in 37C water bath and vortex vigorously. Repeat freeze/thaw/vortex for 3 more cycles (4 cycles total). Do not let virus supernatants warm up during thaw steps.
9. Spin samples (5 min/setting 1) and collect supernatant. Store supernatant at -80 C or proceed to part III.

III. *Second round of infection*:

10. Infect 15 confluent plates using the virus collected in step 9, and repeat infection/collection as described in steps 4-8.
11. For final collection, use 1-2 ml PBS as in step 7, spin cells down with benchtop centrifuge at high speed 10k g for 10 min. Aliquot supernatant and store at -80C.

Note that this virus is not purified, which is usually OK for most application. The CsCl purification protocol can be found in the scanned protocol.

Titration of adenovirus using GFP in 293T cells

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Background

Knowing the titer of the recombinant virus is critical to ensuring good results with your gene expression or knockdown studies.

There are two main types of viral titer.

- **Physical titer**: this is simply the concentration of viral particles in your viral solution and usually expressed as the number of viral particles per mL (VP/mL)
- **Functional titer** or **Infectious titer**: this is the concentration of infectious particles and must be measured after infection of a cell. Functional titer may be expressed in the form of transduction units per mL (TU/mL), plaque-forming unit per mL (pfu/mL), or infectious units per mL (ifu/mL), depending on the viral vector and assay methods.

Functional titer is always a more accurate measurement because it measures how much virus actually gets into the target cell. However, functional titer usually takes much longer to determine and is sometimes not practical. In such cases, functional titer may be estimated from a physical titer measurement. Functional titer will always be lower than physical titer (which does not distinguish active from dead viruses), usually by a factor of 10 to 100-fold.

In this protocol, I describe a rapid and reproducible functional titration method using GFP-expressing adenoviral vectors (Hitt et al., 2000). This protocol can be applied to other types of viral vectors such as retro- or lentivirus as long as the viral vector contains an expression cassette for fluorescence marker genes. Note that TU varies depending on the host cell types or on the culture conditions and therefore it is not an absolute quantification but rather a relative quantification. For example, Adeno-Cre virus gives a titer in 293T cells that is higher than in HC11 cells. It is highly recommended to include your control virus in every titration experiment in order to measure the relative concentration of your viral stocks.

Procedure

Day 1.

On the day before titration, split $2x10^5$ 293T cells per well of a six-well plate. If you are not sure about your approximate viral titers, you need a serial dilution of viral solution, so prepare enough wells for that. (You can estimate the adenovirus copies since usually adenovirus can grow up to 10,000 copies per one 293T cell.) You need at least one well as a negative control for FACS and three wells for cell counting.

Day 2.

The wells should be 10-20% confluent on the next day. Count the number of wells in three wells. The cell number should be 2.0-3.0x10^5/well.

Infection

1. Prepare adenoviral solutions by adding growth medium up to 200 μL in total.
2. Aspirate medium from the cells and add adenoviral solution. Rock the plate to ensure that the entire surface area is covered.
3. Incubate the cells for 60 min in CO_2 incubator, rocking the plate every 15 min.
4. after 60 min, add 2 mL of growth medium.
5. Incubate for 48 h.

Day 4.

After 48 h, visually inspect your cells on a fluorescent microscope to see whether the infection worked. Determine the % of fluorescent cells by FACS and calculate the titer in transduction units per mL (TU/mL) by using the following formula; [cell number at time of infection x (% fluorescent cells/100) x 1000 / μL of viral stock]. For accurate titering , only use infections that result in fewer than 15% fluorescent cells, as these represent one viral integration per cell.

Notes

An example of lentivirus titration experiment is attached to this protocol

References

Hitt, D.C., Booth, J.L., Dandapani, V., Pennington, L.R., Gimble, J.M., and Metcalf, J. (2000). A flow cytometric protocol for titering recombinant adenoviral vectors containing the green fluorescent protein. Molecular biotechnology *14*, 197-203.

(This protocol is a quick than what I used. Recommended).

An example of lentivirus titration experiment (GFP-
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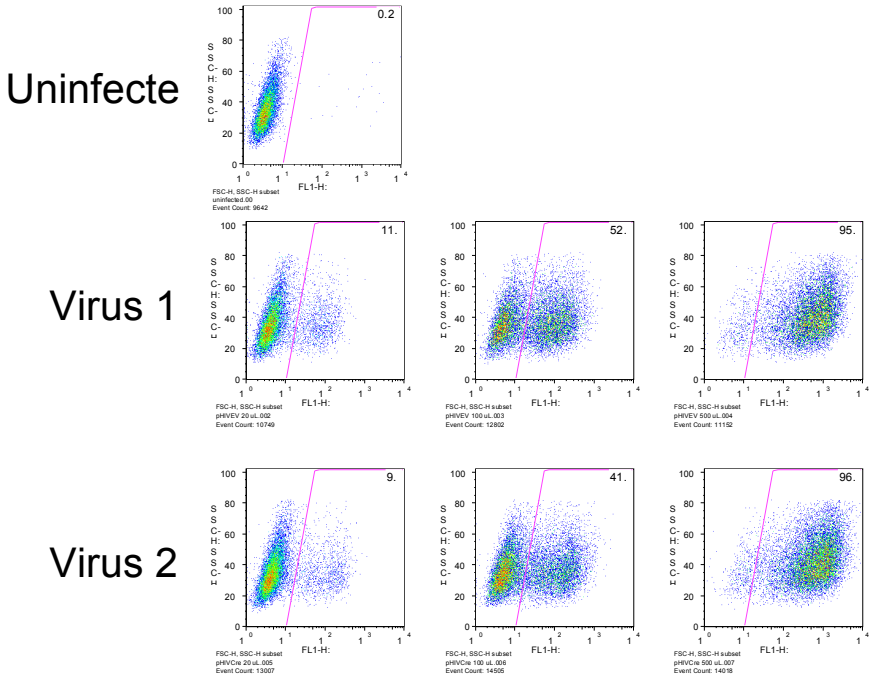

TU = [cell number at time of infection x (% fluorescent cells/100) x 1000 / mL of viral stock]
cell number at time of infection = 3.0×10^5
TU (Virus 1) = 3.0×10^5 x 11.8/100 x 1000 / 20 = 1.77×10^5
TU (Virus 2) = 3.0×10^5 x 9.1/100 x 1000 / 20 = 1.37×10^5