YCCEH Protocols

RETROVIRUS PRODUCTION

(Lipofectamine 3000 Method)

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Reagents

DMEM medium: high glucose with pyruvate, Thermo Fisher, #11995-065
Heat inactivated FBS: Thermo Fisher # 10438-026
PSG (Pen Strep Glutamine, 100x): Thermo Fisher # 10378-016
Lipofectamine 3000: Thermo Fisher # L3000015 (this is for 1.5 ml; smaller sized packages are also available)
1M HEPES buffer (pH7.2 to 7.5): Thermo Fisher #15630-080
DMEM medium: DMEM+10% FBS +1%PSG
VCM: DMEM medium+20mM HEPES (49ml of DMEM medium+1ml of 1M HEPES)
0.45um syringe filter: VWR #28145-505
Packaging plasmids: please refer to Addgene https://www.addgene.org/viral-vectors/retrovirus/. We use the packaging plasmids from the Weinberg lab.

Retrovirus production

Overview

Infection with retrovirus is a useful technique to transduce genetic materials stably into hematopoietic cells, especially those that are actively dividing.

We use 293T cells to package retrovirus. This protocol uses a co-transfection of the viral construct together with VSVG (a.k.a. VSV-G) and gag/pol packaging plasmids. VSV-G is an envelop glycoprotein from vesicular stomatitis virus, and viruses packaged with VSV-G can in theory recognize and infect all mammalian cells.

Compared to contemporary lentivirus vectors, commonly used retroviral vectors tend to be smaller in size, and tend to produce virus with higher titer compared with lenti-vectors. Thus retrovirus could be sometimes advantageous when the genetic payload is large. However, while lentivirus is capable of entering the nucleus and integrating into the genomes of non-dividing cells, retrovirus integrates preferentially into dividing cells.

We routinely prepare retrovirus in 10cm tissue culture plates. It is possible to make virus in 6 well plates, if only a small quantity of virus is needed. The following protocol is for 10cm dishes. Scale accordingly if using a different-sized culture.

293T has many different “strains”. The cells with the same name from different labs can have large differences in viral production efficiency. It is very important to get a good strain of 293T cells by testing different batches. Because 293T cells attach loosely to dishes, good 293T cells for the virus-packaging purpose are often those that can still attach to the plate at high confluency.

293T cells should be cultured in DMEM with high glucose, L-glutamine and sodium pyruvate, plus 10% FBS and 1% L-glutamine and 1% Pen/Strep. DMEM without sodium pyruvate lowers the viral titer.

The half-lives of retrovirus at 37C or room temperature are relatively short. So avoid exposing virus to high temperatures whenever possible.

CAUTION: Proper Biological Safety precaution should be followed. Bleach pipets, tips and plates that have been in contact with virus.

Procedure

Plate 293T cells

Split a confluent 293T plate (10cm) the night before transfection. Count cells before plating. The exact concentration needs to be tested for each batch of 293T cells. We routinely plate 3x10e6 cells per plate as a starting point for optimization.

Transfection

We normally transfect in late afternoon (~5pm).

Prepare plasmid mixture containing 2.5ug of the retroviral construct, 2.5ug of the gag/pol packaging plasmid and 2.5ug of the VSVG packaging plasmid.

For each transfection, prepare two Eppendorf tubes of 0.35 ml of serum-free DMEM medium in TC hood. Leave tubes in the hood till temperature reaches room temperature. (Note: Optimem can be used in place of the serum-free medium).

Add the plasmid mixture into one of the tubes of 0.35ml of serum-free DMEM. Mix by tapping the tube. Add 15ul of P3000 reagent from the Lipofectamine Kit. Mix well. Brief spin to collect volume at the bottom of the tube.

Add 22.5ul Lipofectamine 3000 reagent into the second tube of 0.35ml of serum-free DMEM. Make sure to cap the Lipofectamine 3000 reagent tube immediately and tightly after use. Lipofectamine 3000 should be added with a P200 tip directly into the medium rather than on the tube wall. Pipet up and down once using the P200 tip. Use a P1000 pipet (set to 350ul) to gently pipet up and down once or twice to mix well. Let the tube sit in room temperature for 2mins.

Drop-wise add the Lipofectamine 3000 mixture to the plasmid mixture, using a 1ml tip. Pipet up and down gently once or twice to mix. Let the tube sit in room temperature for 10 mins.

Using a P1000 tip, gently mix the plasmid/Lipofectamine mixture once, and drop-wise add the mixture into a cultured 293T plate. Distribute the droplets across the whole plate. Tilt the plate gently several times to mix.

Adding VCM

In early morning after transfection (~9am), add 15 ml VCM (viral collection medium) to the transfected cells.

Note: 293T cells can easily detach from the bottom of the plate. So avoid adding medium directly onto the cells. Add slowly onto the side rim of the plate. Tilt plate carefully to mix.

Harvest Virus

In the afternoon of the day post-transfection (~5pm), aspirate out all the medium from the plate. Change medium into VCM collection medium for the first collection.

We harvest a total of 2 days, with two harvests each day. We use 7.5ml medium for overnight harvest (harvest ~9am in the morning) and 5ml medium for daytime harvest (~5pm in the afternoon). To harvest the virus, simply pipet the medium out of the plate and into a collection tube. Add new VCM for the next harvest, again to the side wall.

After harvesting the overnight virus, keep the collection tube on ice. We normally put the tube of virus in an ice bucket, with the ice bucket placed into a 4C fridge.

Combine daytime harvest with previous overnight harvest (alternatively, we can combine all four harvests together). Spin down at 1000g for 5-10 mins in a 4C centrifuge to remove 293T cells. Alternatively, after the spin-removal of 293T cells, an additional step of filtering by 0.45 micron filter can be added. For filtering, use a properly sized syringe, e.g. 10ml, get the virus supernatant into syringe, attach filter to syringe, and filter into a new tube. Spin-removal is normally sufficient, which also minimizes virus loss and reduces hazardous waste. However, if freshly prepared virus will be used in experiment (i.e. virus without freezing), the filter-based method is recommended. With only spin-removal of 293T cells, we have seen cases in which 293T cells contaminated the infection culture, indicating that spin-removal of 293T cells is not always 100% complete. However, if the virus has been frozen in -80C before thawing and use, we have never observed 293T cell contamination with the spin-removal approach.

Aliquot supernatant into 3ml cryo vials. Label the name of the viral construct, name of preparer, date, and day of harvest on tube. Store in -80C.

Virus can remain active in -80C storage for years (we have used >10 year old viruses successfully). Freeze-thaw may reduce virus titer a little, but we do not see major decreases.