Plasmid Amplification

Plasmid DNA from **abm** inc. is supplied in 10mM Tris (unless otherwise requested) and intended for direct transformation into an authentic DH5a *E.coli* strain. To amplify any plasmid received from **abm**, transform into DH5a competent cells, pick a single colony and perform a mini-plasmid preparation as usual (it is better not to amplify the plasmid in protein expression strains of *E.coli,* since they are not always well suited to plasmid extraction and purification). Always check with the provider or the appropriate reference to determine the antibiotic selection for the plasmid. Resistance genes included are usually ampicillin, kanamycin, or spectinomycin, but can also include tetracycline or chloramphenicol in some cases. All of **abm**'s pLenti vectors are high copy plasmids.

Subcloning Efficiency DH5a competent *E.coli* cells

- Use **abm**'s ProClone™ Competent Cells (Cat. E003) for best results
- Cells will be provided in 4 X 1.25 ml aliquots and are stored at -80°C.
- The cells will need to be aliquoted to avoid freeze-thaw cycles.
- Thaw a vial on wet ice. Pre-chill the fresh tubes before adding cells to the aliquots.
- Mix the cells after thawing, by gentle inversion.
- Promptly aliquot into 50 µl aliquots and re-freeze in dry ice/ 95% ethanol bath.

Transformation Protocol

1. Thaw an aliquot of ProClone™ Competent DH5a cells on wet ice.
2. Add 1 µl of plasmid. If plasmid concentration is given, dilute to 10 ng/µl and use 1 µl. Mix by tapping the tube gently. Leave on ice for 30 mins.
3. Heat-shock for exactly 45 seconds in a 42°C water bath.
4. Place the tube back on ice for 2 mins.
5. Add 150 µl sterile LB broth, and recover for 1 hour in an incubated shaker set at 37°C, 240 rpm.
6. Spread entire volume of cells on an LB agar plate containing the appropriate antibiotic (see below for concentrations*).
7. Incubate at 37°C overnight (around16 hours) to allow colonies to form. If the colonies are too dense, plate 1µl cells in a 100 µl pool of LB on a fresh LB + antibiotic plate.
8. Inoculate 4-10 ml of LB broth containing the appropriate antibiotic with a single picked colony. Grow overnight (16-18 hours) in an incubated shaker set at 37°C, 240 rpm.
9. Isolate plasmid by a mini-prep protocol, as standard.

***Antibiotic Selection:**
KanR: 50 µg/ml Kanamycin
AmpR: 100 µg/ml Carbenicillin/Ampicillin
SpecR: 50 µg/ml Spectinomycin
TetR: 12.5 µg/ml Tetracycline
CamR: 25 to 34 µg/ml Chloramphenicol

1-866-757-2414 (Toll Free)
604-247-2416 (Local)
Order: order@abmGood.com
Website: www.abmGood.com
Applied Biological Materials Inc.
8 - 13520 Crestwood Place,
Richmond, BC, CANADA V6V 2G2

Lentivirus Packaging Protocol

The following protocol allows for the production of recombinant lentiviral particles up to a 10^6 IU/mL titer. We recommend including a negative control (without DNA or transfection reagents) in your experiments to help evaluate your results.

Before starting the lentiviral packaging protocol, please ensure that you have an adequate volume of expression DNA (10 µg plasmid /10 cm dish). A DNA amplification step will usually be required using standard bacterial transformation protocols. We recommend using *E.coli* DH5-alpha strains for amplification of all DNA plasmids from **abm**, this strain has been tested to produce high yields of plasmid for lentiviral packaging with minimal risk of recombination occurring.

Protocol

DAY 1:
1. In the afternoon, seed ~1.2 x 10^7 293T cells in a 10 cm dish.

DAY 2: (Carry out steps 2 - 6 in the morning on the day of transfection)
2. Check to make sure the cells are 70-80% confluent.

3. a) **For each 10 cm dish** prepare the transfection complex as follows:
Solution A: Dilute 20 µg DNA plasmids (10 µg expression vector and 10 µg of **abm**'s Second Generation (LV003) or Third Generation (LV053) Packaging Mix) in 1 mL serum-free, antibiotic-free medium. Solution B: Dilute 80 µL of LentiFectin™ Transfection reagent (G074) in 1 mL serum-free, antibiotic-free medium.

b) Incubate both solutions at room temperature for 5 minutes.
c) Mix Solutions A and B together well and incubate at room temperature for 20 minutes. This will create the transfection complex.

4. Add 4.5 mL serum-free medium to the transfection complex.

5. Remove medium from the cells in the 10 cm dish.

6. Add the complete transfection complex from step 4 to the cells and incubate at 37°C for 5-8 hours. Avoid dislodging the cells by gently adding the mixture against the side wall of the dish.

7. Add 0.65 mL FBS to the 10 cm dish and incubate at 37°C overnight.

Continued

DAY 3:
8. Remove the transfection medium from the cells.

9. Add 10 mL complete culture medium to the cells.

10. Incubate at 37°C for 24 hours.

DAY 4 (Harvest):

11. Collect the supernatant medium from the culture dish.

12. Centrifuge the supernatant at 3000 rpm for 15 minutes at 4°C to pellet cell debris.

13. Transfer the cleared supernatant to a fresh tube. Filter the cleared supernatant with a low-protein binding 0.45 µM sterile filter.

14. The viral titer of the first harvest is approximately 10^6 IU/mL. The filtered supernatant will be ready for *In vitro* infections or further concentration and/or purification. Alternatively, it can be stored at -80°C as viral stock for future applications. Aliquotted volumes are preferred for long term storage to reduce the loss of viral titer through multiple freeze-thaw cycles.

15. A second harvest can be carried out by adding 10 mL of complete medium to the cells after the first harvest and incubating at 37°C for a further 24 hours. The first harvest can be stored at 4°C overnight to allow the second harvest to be added to it the following day (freezing the supernatant would result in a greater loss of titer).

16. Collect the second supernatant on Day 5 (as in steps 11-13) and combine this with the first harvest.

Note: Expression of the VSVG glycoprotein causes 293T cells to fuse, resulting in the appearance of large, multinucleated cells known as syncytia. This morphological change is normal and does not affect the production of the lentivirus.

17. For viral titers that are 10^6 IU/mL and higher, you can quickly and easily titer your virus preparation using the qPCR Lentivirus Titer Kit (LV900) available from **abm**.

In addition, our Ultra-Pure Lentiviral Purification Kit (LV998) will allow you to concentrate the virus to a higher titer if desired.

Lentiviral Infection of Target Cells

Important notes: The transduction efficiency of mammalian cells varies significantly under different experimental conditions. This includes virus concentration, exposure time to the virus and growth area of the well or plate used for the infection.

To determine the viral concentration required to provide the desired multiplicity of infection (MOI) for your target cells, it is advisable to perform several test transductions with reporter viral particles (e.g. GFP Control LV006 or ß-gal Control LV007 lentiviruses) at a range of different volumes such as 1 µl, 5 µl, 10 µl, and 100 µl. Results from these preliminary tests can be used to determine an optimal concentration that will yield the highest percentage of successfully infected cells.

Downstream assays should be carried out 48-72 hours following transduction if no antibiotic selection is used. The decision to directly assay without selection will depend on the transduction efficiency and proliferation rate of your target cells and also the biological assay(s) you will be performing.

For cells with a high infection efficiency (e.g. HEK 293, HT1080, HeLa, MDA-MB-468 cells etc) most biological assays can be performed without a need for selection. For cells that are more resistant to infection, it is desirable to select only the clones that stably express the lentivector construct for downstream experimental assays.

Protocol

The following protocol has been provided as a general guideline only, to be used as a starting point for determining optimal conditions for target cell transduction:

1. Plate the target cells in a 24-well plate, 24 hours prior to viral infection at a density of 0.5×10^5 cells per well. Add 0.5 ml of complete optimal medium (with serum and antibiotics if required) and incubate the cells at 37°C with 5% CO_2 overnight.

Note: It is possible to use other plate formats for transduction. In this case, the amount of cells should be adjusted depending on the growth area of the well/plate.

2. Prepare a mixture of complete media with polybrene at a concentration of 8 µg/ml. Remove the growth media from the wells and replace with 0.5 ml of the polybrene-media-mix per well (adjust volume as necessary if using a different size plate). If the transduction efficiency of the target cells is low, add in ViralPlus Tansduction Enhancer G698 at 1:100 (or your own optimized dilution ratio).

3. Once an effective MOI has been determined for the target cells through preliminary test infections, use the appropriate volume of virus to infect your cells. Cas9 and sgRNA lentiviruses can be used to co-infect target cells at the same time.
You should include a transduction well with a positive GFP control virus and an appropriate blank control viral construct. Leave one well of uninfected cells as an additional standard control. Following the infection, incubate the cells at 37°C with 5% CO_2 overnight.

Continued

4. Remove the culture medium and replace with 1 ml of complete medium. Incubate the cells at 37°C with 5% CO_2 overnight.

5. The following day, split the cells 1:3 or 1:5 (depending on the growth rate of your target cells) and continue incubating for 48 hours in complete media.

6. The infected cells can then be selected for stable expression using appropriate antibiotic selection at a minimum concentration, as determined by a killing curve. Genome editing can then be assayed by a number of techniques, including Western blot, sequencing or Surveyor assay.

Amplification Protocol

Pre-made Adenovirus Amplification

Pre-made adenovirus from **abm** is provided as a seed-stock only and will require further amplification for downstream *in vitro* transduction. Large-scale virus production and purification will be necessary for *in vivo* injections.

IMPORTANT INFORMATION
It is strongly recommended to always amplify one adenovirus seed- stock at a time and in different culture hoods and incubators if possible. Where only one set of equipment is available, amplify the viruses sequentially and use UV radiation for 30 minutes in-between working with each virus. As cross-contamination when working with two or more adenoviruses is a major risk, we also recommend using separate trypsin and medium containers for each virus.

Protocol

1. When you receive your recombinant adenovirus, make two to three aliquots and use one for amplification in 293 cells. Freeze the remaining aliquots in -70°C as a seed stock for future use.

2. Amplify your adenovirus in HEK 293 cells, plated at 60-70 % confluency. For a 60 mm dish, infect the cells with 70 µL of the adenovirus, for a 100 mm dish, infect the cells with 200 µL of virus.

3. When more than 95% of 293 cells are detached from the dishes, collect both the cells and medium into a large falcon tube.

4. Freeze (in a -70°C freezer or dry ice / ethanol) and thaw (in a 37°C water bath) the collection three times.

5. Pellet the cell debris by centrifugation at 3,000 rpm at room temperature for 10 minutes.

6. Transfer the supernatant into a fresh tube. Store at 4°C for short-term use (two to three weeks) or add glycerol to a final concentration of 10% and freeze at -70°C (stable for one to two years).

7. **Transduction Procedure:** If the virus is to be used in an *in vitro* transduction, double CsCl purification is not required as the viral supernatant will provide 100% gene transduction efficiency in most human cell lines. For *in vivo* studies purification is essential to remove defective particles, cell debris, and residual media components, since these contaminants can induce significant immune responses. In addition, CsCl purification will concentrate the virus to a level suitable for *in vivo* injections.

1. Prepare target cells in a 6-well plate or 10 cm at 70% confluency one day prior to transduction.

1-866-757-2414 (Toll Free)
604-247-2416 (Local)
Order: order@abmGood.com
Website: www.abmGood.com
Applied Biological Materials Inc.
8 - 13520 Crestwood Place,
Richmond, BC, CANADA V6V 2G2

2. Aspirate the culture medium and overlay with viral culture supernatant (1ml for a 6-well plate and 4-5 ml for 10 cm dishes) to cover the cells for one hour in an incubator.

3. Remove the media containing the virus and replace it with fresh complete media.

4. Genome editing can be evaluated 48-72 hours after transduction by different assays, such as Western blot, sequencing or Surveyor assay.

For any further queries, please contact our technical support team at technical@abmgood.com and we will get back to you promptly.