

Chapter 2

Mammalian Expression Systems and Transfection Techniques

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Abstract

To delineate the function of a single ion channel subtype amongst the multitude that normally constitutes a signalling pathway, it is often insightful to study the function or signalling of that one ion channel in the absence of the others. Mammalian cell lines that do not normally express the gene of interest can be manipulated to do so via plasmid DNA expression vectors. However, large and highly charged molecules like DNA cannot passively diffuse through cell membranes. Therefore introducing nucleic acid into mammalian cells may involve introducing pores transiently into the cell membrane to allow the passage of circular plasmid DNA. This is relatively easily carried out using cationic lipids that form liposomes around the DNA and fuse with the cell membrane to introduce the DNA inside the cell. Alternatively, a highly successful mechanism for introduction of DNA involves utilizing viral vectors. These take advantage of the molecular mechanisms that viruses have evolved to efficiently transport their genome inside cells. Lipid-based transfection techniques and adenoviral delivery of plasmids encoding large genes (such as ion channel genes) for expression in mammalian cells are the focus of this chapter.

Key words Transient transfection, Stable transfection, Adenovirus, Transfection efficiency, Reporter gene, Bicistronic vector

1 Introduction

Since many ion channels are regulated by diverse posttranslational modifications (such as phosphorylation, glycosylation, disulfide bond formation) that can differ between species, in order to study mammalian ion channel function in a heterologous system, it is beneficial to use a mammalian expression system. Mammalian cells can be transfected with plasmid DNA encoding ion channel genes. Plasmid DNA may either be introduced into the cells in a transient fashion or may integrate into the cell genome. Cells that have incorporated the plasmid DNA into their genome can be selected via the use of antibiotic resistance genes to generate stable clones that express a protein of interest. This chapter focuses on the methods of transient and stable transfection by lipid-based

transfection and transient expression by adenoviral delivery. Three methods of assessing transfection efficiency and identifying transfected cells are also discussed.

1.1 Using Reporters to Analyze the Efficiency of Transfection

The efficiency of transfection is a major concern for many applications and should be monitored using a reporter, such as a fluorescent protein, e.g., enhanced green fluorescent protein (eGFP), to confirm the transfer of nucleic acid to the cell. This can be achieved by (1) transfecting two separate plasmids, one of which encodes eGFP. This is the weakest of the three options presented here; although it is likely that if one plasmid was able to transit through a particular cell membrane then both plasmids were able to do so, it cannot be guaranteed (see Note 1). (2) Generation of a fusion protein, using a plasmid such as pEGFP-N1 or pEGFP-C1 (Clontech), in which your protein of interest is tagged with eGFP (as one continuous protein). Careful consideration should be given as to the position of the eGFP relative to the protein of interest (i.e., N-terminal or C-terminal) since the 27 kDa eGFP may affect folding, localization, or function of your target protein. (3) Another option would be to use a bicistronic vector (generates a single mRNA that encodes two proteins), such as pIRES2-eGFP (Clontech). This vector contains an internal ribosomal entry site (IRES) sequence, a nucleotide sequence that promotes translation initiation in the middle of an mRNA and thus allows the simultaneous expression of the protein of interest and eGFP (as a separate protein) from the same mRNA transcript. With this method eGFP expression can be used to determine efficiency of transfection and identify those cells that are positively transfected (since all cells expressing the eGFP were successfully transfected) without the eGFP affecting the folding of the protein of interest. One other important consideration is that the expression of any reporter may affect the expression of other proteins or the function of the cell, thus control transfections should be carried out in which the eGFP (in the absence of your protein of interest) is expressed (see Note 2).

1.2 Transient Versus Stable Transfection

The type of transfection method used, the size of the DNA plasmid to be transfected, the amount of DNA transfected relative to the amount of reagent, and the number of cells plated will all affect the efficiency of transfection. Many ion channel genes are relatively large, often leading to low-transfection efficiencies in mammalian cells. Particularly in cases where the efficiency of transfection is an issue, or to standardize experiments, you may want to consider generating a stable cell line, in which the plasmid has integrated into the genome of the cell. This means that the cell line stably expresses your protein(s) of interest through successive rounds of division. With transient transfections, expression of the proteins will reduce as the plasmid is lost through cell division.

For this reason, cells are often used for experiments 24–48 h after transfection (division approximately once every 24 h in many mammalian cell lines). Although more time consuming initially, generating stable cell lines can save a large amount of time and money throughout the project and may also lead to more consistent results. Plasmids such as pEGFP-N1 and pIRES2-GFP contain a neomycin resistance gene for selection of stably transfected cells. Selection of stable clones is achieved by incubating the cells with G418 (also known as geneticin) over a period of weeks. Cells that have not integrated the plasmid into the genome die following extended exposure to G418. Individual cells expressing the reporter gene can then be picked and diluted to single cells that then undergo clonal expansion.

1.3 Adenoviral Delivery

Viral vectors permit high levels of transgene expression in many cell types, often without affecting cell viability. Since the adenoviral DNA does not integrate into the genome and is not replicated during cell division, adenoviral delivery mediates transient expression of transgenes. The adenoviral DNA vector contains a portion of the viral genome plus a gene of interest cloned into a multiple cloning site inserted into the early region 1A (E1A) of the genome. Recombinant adenoviral vectors are generated using this viral DNA vector and a packaging cell line (e.g., HEK293, which have been stably transfected with the E1A region of the adenoviral genome). Via a method adapted from He and colleagues (1), we have previously successfully prepared adenoviral vectors to transiently express genes and reporters in cell lines (2, 3) and primary cells, including neurons (4) and cardiac myocytes (5). The gene of interest is cloned into the pAdTrack-CMV vector, linearized, and transformed into an *Escherichia coli* strain BJ5183 that expresses pAdEasy adenoviral vector (pAdEasy-1). The recombinant adenoviral construct is then transfected into a packaging cell line (e.g., HEK293) for virus production. During the generation of the HEK293 cell line, normal human embryonic kidney cells were transformed with adenovirus five DNA and this cell line can be utilized for the propagation of adenoviral vectors. Since adenoviruses are pathogenic it is important to use adenoviral vectors in which certain genes are deleted so that the viruses are unable to replicate after entering a cell. HEK293 can be safely used in combination with the adenoviral vector pAdEasy, in which the E1 and E3 genes have been deleted (1). Using this system viral production and transgene expression can be monitored with the reporter eGFP.

In this chapter, we outline the lipid-based methods used to transfect mammalian cells transiently and following on from this how to generate stable cell lines. We then outline how to prepare adenovirus particles, which can be used to infect mammalian cells to express genes of interest.

2 Materials

1. Cell culture medium: Dulbecco's Modified Eagle Medium (DMEM), 10% fetal bovine serum, 2 mM glutamine, streptomycin (10 g/L), and penicillin (10 g/L); pre-warmed to 37°C.
2. Serum-free media (e.g., Opti-MEM).
3. Lipid transfection reagent [e.g., Lipofectamine 2000, Lipofectamine LTX (Invitrogen)].
4. Cell culture plates.
5. Phosphate-buffered saline (PBS).
6. Sterile, round bottomed 14 mL centrifuge tubes.
7. Purified plasmid DNA (e.g., pIRES2-GFP, pAdTrack-CMV).
8. Cell line for transfection, e.g., HEK293, CHO, Neuro2A
For stable transfection:
9. G418 (Sigma).
10. 96-well cell culture plates.
For adenovirus preparation:
11. Restriction enzymes *PmeI*, *PacI*.
12. Bacterial strain BJ5183 containing pAdEasy-1.
13. Bacterial strain XL1 Blue.
14. Electroporation cuvettes, electroporator (option to use chemically competent cells and heat shock for electroporation).
15. Luria-Bertani Media (LB Broth: Tryptone 1.0%, Yeast Extract 0.5%, NaCl 1.0%)+kanamycin (10 µg/mL) or ampicillin (25 µg/mL). For plates, 10% agar.
16. Tris-HCl pH 8.0 0.1 M.
17. Sodium deoxycholate 5%.
18. Magnesium chloride 2 M.
19. DNaseI (100 mg DNaseI in 10 mL of 20 mM Tris-HCl pH 7.4, 50 mM NaCl, 0.1 mM DTT, 0.1 mg/mL BSA, and 50% glycerol).
20. Cesium chloride 1.5 g/mL (90.8 g CsCl and 109.2 g 10 mM Tris pH 8).
21. Cesium chloride 1.35 g/mL (70.4 g CsCl and 129.6 g 10 mM Tris pH 8).
22. Cesium chloride 1.25 g/mL (54 g CsCl and 146 g 10 mM Tris pH 8).
23. Polyallomer tubes.
24. Dialysis buffer (10 mM Tris-HCl pH 8, 10% glycerol).
25. Slide-a-lyzer dialysis cassette.

3 Methods

3.1 *Transient Transfection*

1. Seed cells at a density of 1.5×10^6 cells per well of a 6-well plate 24 h prior to transfection.
2. Dilute 10 μg of plasmid in 250 μL of Opti-MEM (mix A).
3. Dilute 30 μL of Lipofectamine 2000 in 250 μL Opti-MEM (mix B).
4. Incubate at room temperature for 20 min.
5. Add mix A to mix B.
6. Incubate for 25 min at room temperature.
7. Wash the cells with PBS.
8. Add the mix A + B to cells.
9. Incubate for 1 min with gentle agitation.
10. Add 500 μL of Opti-MEM medium.
11. Incubate for 1 h at 37°C .
12. Add 1.5 mL of DMEM: 10% fetal bovine serum and glutamine added—NO ANTIBIOTICS.
13. Incubate the cells for 24 h at 37°C 5% CO_2 .
14. Change medium or add 1 mL of DMEM: 10% fetal bovine serum and glutamine WITH antibiotics (if required).
15. After 24 h change medium and check fluorescence under microscope.

3.1.1 *Optimization of Transfection Efficiency*

Cell lines that transfect easily and show high levels of transfection efficiency (>80%) using this method include human embryonic kidney 293 (HEK293) and Chinese hamster ovary (CHO). Other cell lines that show reduced transfection efficiencies may be required for use, e.g., the mouse neuroblastoma cell line Neuro2A. In this case, it is extremely important to optimize transfection; some hints for this are included below. We tested Lipofectamine LTX (which is suggested by the manufacturer for high-efficiency transfection of Neuro2A cells) and Lipofectamine 2000 (which is not specifically recommended by the manufacturer for transfection of Neuro2A cells). Based on the original protocol from both products two different transfections were tested. For each, a control transfection was carried out with the “empty plasmid” pEGFP-N1 (4.7 kb) (control plasmid) and the test plasmid containing the cloned gene of interest in pEGFP-GOI (11 kb).

- A. Lipofectamine 2000, following the manufacturer’s protocol for general transfection of 1×10^6 murine cells in a 6-well plate. The protocol was as above except for:
 1. Transfection with 4 μg DNA and 10 μL Lipofectamine 2000.

2. Incubate mix A and B at room temperature for 5 min.
 3. Incubate mix A+B with cells at room temperature for 1 h.
 4. Add 1 mL of DMEM 10% FBS+ glutamine without antibiotics.
 5. Incubate cells for 24 h at 37°C 5% CO₂.
 6. After 24 h add 1 mL of DMEM with antibiotics.
- B. Lipofectamine LTX, following the manufacturer's protocol for transfection of 2.5×10^5 Neuro2A cells in a 6-well plate.
1. Aspirate media and wash cells with PBS.
 2. Add 1.5 mL DMEM: 10% FBS and glutamine without antibiotics.
 3. Incubate 1.25 µg DNA in 500 µL of Opti-MEM.
 4. Incubate at room temperature for 5 min.
 5. Add 20 µL Lipofectamine LTX to the mix.
 6. Incubate at room temperature for 25 min.
 7. Add mix to cells.
 8. Incubate cells for 24 h at 37°C 5% CO₂.
 9. Change medium after 24 h with DMEM with antibiotics.

The transfection efficiencies achieved with these two protocols of Neuro2A cell transfection with pEGFP-N1 and pEGFP-GOI are given in Table 1. Increased efficiencies for transfection were identified by optimization of the following steps:

1. Increased number of cells seeded (2×10^6 cells in a 6-well plate) (step 1 of Subheading 3.1).
2. Increased Lipofectamine 2000 (30 µL was the optimal volume; the results were similar with 35 µL but lower with 25 µL) (step 3 of Subheading 3.1).
3. Increased time of separate incubation for mix A and mix B in Opti-MEM (up to 25 min) (step 4 of Subheading 3.1).
4. Increased time of incubation after transfection (2–3 h) (step 11 of Subheading 3.1).

Table 1

Transfection efficiencies vary with plasmid size and reagent used

Lipofectamine + plasmid	Efficiency of transfection (%)
Lipofectamine 2000 + plasmid pEGFP-N1 4.7 kb (empty, control)	20
Lipofectamine 2000 + plasmid pEGFP-GOI 11 kb	15
Lipofectamine LTX + plasmid pEGFP-N1 4.7 kb (empty, control)	15
Lipofectamine LTX + plasmid pEGFP-GOI 11 kb	5

Table 2
Transfection efficiencies vary with ratio of DNA:lipophilic reagent and incubation time

Plasmid size (kb)	Incubation time	DNA (μ g)	Lipofectamine (μ L)	Efficiency of transfection (%)
4.7	10	10	30	10–15
4.7	20	10	30	15–20
4.7	25	10	30	20–30
4.7	30	10	30	20–30
4.7	35	10	30	10–15
11	10	10	30	5–10
11	20	10	30	15–20
11	25	10	30	20–30
11	30	10	30	30–35
11	35	10	30	30–35

The efficiencies of transfection for the 11 and 4.7 kb plasmid were both increased to 40% with these changes (see Table 2 and Note 3).

3.2 Stable Transfection and Clone Selection

1. After transfection of Neuro2A cells with Lipofectamine 2000 as above, add G418 to a final concentration of 400 μ g/mL.
2. Incubate for 2 days with G418.
3. Change medium and incubate without G418 for 4–5 days.
4. After 5 days change medium to include G418 to a final concentration of 600 μ g/mL.
5. Increase the concentration of G418 up to a final concentration of 800 μ g/mL for a further 2 weeks (see Note 4).
6. Trypsinize cells and dilute to the appropriate volume to yield single cells in a 96-well plate and allow clones to expand (Fig. 1). To do this perform dilutions in a 96-well plate, starting with 2×10^5 cells in A1 then serially diluting in the first column of the plate by a dilution of 1:2. Perform a second dilution series, beginning from column 1 and serially diluting by 1:2 in columns 2–12. Incubate the 96-well plates in a 5% CO₂ incubator at 37°C.
7. Identify wells containing single clones (generally rows F–H columns 10–12), transfer each clone to a single well in a 24-well plate, and incubate with G418 at a final concentration of 600 μ g/mL for 2 weeks.

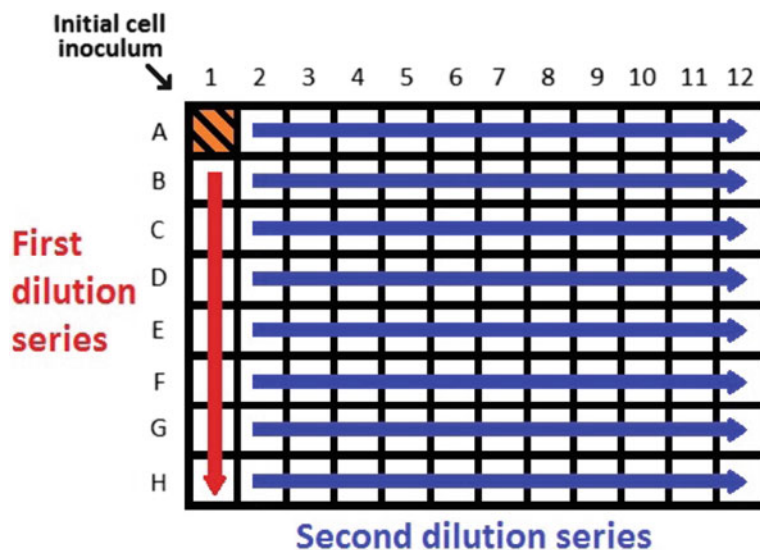


Fig. 1 Dilution of transfected cells to isolate single clones. Perform dilutions in a 96-well plate, starting with 2×10^5 cells in A1 then serially diluting in the first column of the plate by a dilution of 1:2. Perform a second dilution series, beginning from column 1 and serially diluting by 1:2 in columns 2–12. Wells containing single cells are generally within the bottom right-hand corner of the plate, e.g., rows F–H, columns 10–12

8. Transfer single clones to a single well in a 24-well plate and allow to proliferate for 5 days (see Note 5). Treat with G418 at 400 $\mu\text{g}/\text{mL}$ to maintain stably transfected cells.

3.3 Adenovirus Particle Preparation

3.3.1 pAdEasy Recombination

1. Digest 0.5–1.0 μg of pAdTrack-CMV plasmid with *PmeI* and purify by agarose gel purification. Resuspend DNA in 50 μL purified water.
2. Set up 5 mL overnight culture of BJ5183 (pAdEasy-1) in LB+ampicillin at 37°C shaking at 225 rpm on an orbital shaker.
3. Grow for several hours until reaching an OD of 0.6–0.8.
4. Pellet cells at $2,000 \times g$ 10 min at 4°C.
5. Resuspend in 10 mL ice cold water.
6. Pellet cells at $2,000 \times g$ 10 min at 4°C.
7. Resuspend in 1 mL of ice cold water and transfer to Eppendorf tube.
8. Pellet cells in microfuge for 10 s.
9. Wash pellet two times with ice cold water.
10. Pellet cells in microfuge for 10 s.
11. Resuspend cells in 25 μL of digested DNA.

12. Warm 1 mL SOC media per transformation.
13. Place cell suspension in 2 mm cuvettes.
14. Electroporate at 2.5 kV, 200 , and 25 μ F in a Bio-Rad Gene Pulser T.
15. Immediately transfer cells to 1 mL warm SOC.
16. Incubate at 37°C with shaking for 1 h.
17. Plate onto LB+kanamycin plates and incubate at 37°C overnight.
18. Pick a colony and grow overnight in 5 mL LB+kanamycin at 37°C and prepare DNA by standard miniprep kit (e.g., Qiagen). Identify pAdEasy clones and transform into XL1 Blue.

3.3.2 Transfection of pAdEasy

1. Prepare pAdEasy recombinant plasmid DNA from a 50 mL culture from a colony from step 18 (XL1 Blue transformed).
2. Digest 5 μ g of DNA with *PacI* in 100 μ L volume at 37°C for 1 h.
3. Purify DNA and resuspend in 10 μ L water.
4. Transfect HEK293 cells with purified DNA, as per Subheading 3.1 above.
5. Culture cells for 7–10 days, until the majority of cells are floating and expressing GFP (the adenovirus infected cells will also change morphology and become rounded). Harvest free-floating cells by centrifugation.
6. Resuspend in 1 mL PBS and transfer to Eppendorf tube.
7. Freeze/thaw to lyse cells by transferring from –70 to 37°C for three cycles and vortex.
8. Pellet cell debris at 18,000 $\times g$ for 10 min at 4°C.
9. Transfer supernatant to fresh tube and store lysate at –70°C.

3.3.3 Amplification and Purification of Virus (See Note 6)

1. Take the lysate from step 9 of the previous section and use to infect 2 \times 10 cm tissue culture dishes of HEK293.
2. After 7–10 days the majority of cells will be floating and expressing GFP. Harvest free-floating cells by centrifugation. Resuspend cell pellet in 1 mL PBS per 10 mL media.
3. Freeze/thaw three times in –70°C/37°C, vortexing each time.
4. Pellet cell debris at 18,000 $\times g$ 10 min at 4°C.
5. Transfer supernatant to fresh tube and store at –70°C.
6. Use the second lysate to infect 20 \times 10 cm dishes of HEK293. Lyse cells as above.
7. Use the third lysate to infect 40 \times 15 cm dishes of HEK293 and harvest and purify adenoviral particles as below.

8. Collect cells in 400 mL centrifuge tubes and pellet by centrifugation at $10,000\times g$ for 20 min at 4°C .
9. Resuspend cells in 10 mL 0.1 M Tris pH 8 per 15 cm dish.
10. Pellet cells by centrifugation at $10,000\times g$ for 20 min at 4°C .
11. Resuspend cells in 0.5 mL 0.1 M Tris-HCl pH 8 per 15 cm dish.
12. Add 100 μL of 5% Na deoxycholate per mL of cell lysate.
13. Incubate 30 min at room temperature to produce a clear, highly viscous suspension.
14. Add 10 μL 2 M MgCl_2 and 5 μL DNase (10 mg/mL) per mL of lysate.
15. Mix and incubate 37°C for 60 min, agitating every 10 min. Viscosity should reduce.
16. Centrifuge at $18,000\times g$ for 20 min at 4°C .
17. Prepare a discontinuous cesium chloride (CsCl) gradient in a polyallomer centrifuge tube (do not disturb gradients once formed). To do this, add the following volumes of CsCl of different densities slowly down the side of the tube in this order:
 - (a) Add 0.5 mL of 1.5 mg/mL CsCl.
 - (b) Overlay with 3.0 mL of 1.35 mg/mL CsCl.
 - (c) Overlay with 3.0 mL of 1.25 mg/mL CsCl.
18. Apply 5 mL of supernatant to the CsCl gradient. Prepare further gradients for the remainder of the supernatant.
19. Centrifuge at $28,000\times g$ at 10°C for 1 h. Do not use the brake.
20. Collect viral bands which should be visible as white/purplish bands:
 - (a) Put the tube in a stand in the tissue culture hood above a beaker.
 - (b) Remove several mL of CsCl from the top of the tube to reduce the pressure when you puncture the tube.
 - (c) Place a piece of tape vertically on the side of the tube.
 - (d) Using an 18 gauge needle on a 2 mL syringe, carefully puncture the tube just below the viral band with the bevel of the needle facing upwards (make sure to only puncture one side of the tube, do not push through the other side, insert needle tip to the middle of the tube, directly underneath the viral band).
 - (e) Extract the viral band (approx 1–1.5 mL volume).
21. Transfer the pooled virus to a fresh tube and top up with 1.35 g/mL CsCl.
22. Centrifuge at $28,000\times g$ for 16–24 h at 10°C . Do not use brake.

23. Collect viral band as above in about 0.5–1.0 mL volume and transfer to a slide-a-lyzer dialysis cassette.
24. Dialyse for 3×1 h in 1 L 10 mM Tris–HCl pH 8 10% glycerol in a beaker with a magnetic stirrer.
25. Aliquot and store at -70°C .
26. Titrate virus in cell culture media to infect cells. Test dilutions at 1:50–1:50,000. Incubate cells for 24 h at 37°C 5% CO_2 then monitor GFP expression.

4 Notes

1. Consider increasing the amount of the plasmid encoding your protein of interest compared to the plasmid encoding eGFP (e.g., 2:1 or 3:1) to increase the likelihood of expression of your protein of interest in eGFP-positive cells. This may be especially important if the plasmid encoding your protein of interest is much larger than the plasmid encoding eGFP. For efficient DNA transfection into cells, preparation of high-quality plasmid DNA is essential. It is best to prepare DNA using a commercial kit based on ion exchange chromatography. Removal of endotoxins during the plasmid isolation procedure also enhances transfection efficiency and many companies supply plasmid isolation kits that will remove endotoxin contamination.
2. Transfection can result in changes in gene expression and consequently in altered cellular responses and/or morphology. It is always advisable to compare responses to control transfected cells. In the case of transfection with liposomes, the transfection reagent on its own can affect the cell membrane (and any membrane proteins, such as ion channels); it may therefore also be necessary to include a “transfection reagent” control in experiments, in which transfection reagent is added as per the above protocol but in the absence of plasmid DNA.
3. The incubation time of the single mixes affects transfection efficiency (6). The “empty plasmid” pEGFP-N1 (4.7 kb) (control plasmid) was negatively affected by long incubation times but the larger test plasmid containing the cloned gene of interest pEGFP-GOI (11 kb) showed improved efficiency by increasing the incubation time. Lipofectamine volume was chosen based on the amount of cells transfected and the toxicity to cells (confluence of cells compared with the initial confluence). Finally, decreasing the amount of plasmid reduced efficiency.
4. With the use of G418 for selection of resistant clones it may be important to titrate the antibody concentration depending on the

cell line and plasmid used; concentrations between 200 µg/mL and 1,400 µg/mL are recommended for testing. We also found that G418 reduces proliferation and at low densities the cells were particularly susceptible to death. Therefore we found it unnecessary to split the cells more than once per 2–4 weeks during the time of treatment.

5. We have found that treating the cells with G418 in this single cell state (even very low concentrations) results in high levels of cell death. It is advisable to allow the cells to proliferate before repeating treatments with G418. G418 treatment should be used periodically to maintain selection, since even plasmids that were integrated into the genome can be lost.
6. Exposure to adenovirus (even replication-deficient adenovirus) may cause acute respiratory illness. Adenoviruses are biohazards and appropriate biosafety procedures should be adhered to. Work with virus in a certified Class II biosafety cabinet. Discard of all virus in bleach (1% sodium hypochlorite, made fresh) or similar for at least 15 min and disinfect all plasticware contaminated with virus in bleach for at least 15 min, followed by autoclaving.

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