Chapter 3

Viral Infection for GPCR Expression in Eukaryotic Cells

Antonio Porcellini, Luisa Iacovelli, and Antonio De Blasi

Abstract

This chapter describes the protocol for the preparation of recombinant adenoviruses and infection of target cells to transiently express G protein-coupled receptors (GPCRs) or other proteins of interest. Adenoviruses are non-enveloped viruses containing a linear double-stranded DNA genome. Their life cycle does not normally involve integration into the host genome, rather they replicate as episomal elements in the nucleus of the host cell, and consequently there is no risk of insertional mutagenesis. Up to 30 kb out of the 35 kb of the wild-type adenovirus genome can be replaced by foreign DNA. Adenoviral vectors are very efficient in transducing target cells in vitro and in vivo and can be produced at high titers (>10^{11}/mL). The viral infection has a number of useful features: (1) the efficiency of gene transduction is very high (up to 100% in sensitive cells); (2) the infection is easy and does not physically alter the cell membrane for gene transduction; (3) it is possible to infect cells that are resistant to transfection with plasmids (including nondividing cells); and (4) the viral vectors can be used for infection in vivo (including gene therapy) and can potentially be targeted cell-specifically.

Key words: G protein-coupled receptors, TSH, Viral vectors

1. Introduction

This chapter describes the protocol for the preparation of recombinant adenoviruses and infection of target cells to transiently express G protein-coupled receptors (GPCRs) or other proteins of interest. This technique represents a unique tool as it allows the introduction of exogenous DNA into cellular systems, such as primary cultures that are not suitable for transfection using the traditional approaches.

Viruses are obligate intracellular parasites, designed through the course of evolution to infect cells, often with great specificity for a particular cell type. They tend to be very efficient at transfecting their own DNA into the host cell, and the DNA is expressed to

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produce new viral particles. By replacing genes that are needed for the replication phase of their life cycle (the nonessential genes) with foreign genes of interest, the recombinant viral vectors can transduce the cell type it would normally infect. To produce such recombinant viral vectors, the nonessential genes are provided in trans, either integrated into the genome of the packaging cell line or on a plasmid.

A number of viruses have been developed, but four types are mostly used: retroviruses (including lentiviruses); adeno-associated viruses; herpes simplex virus type 1 (HSV-1); and adenoviruses. Viral constructs for cell infections can be obtained commercially from different sources (see Note 1).

Retroviruses are a class of enveloped viruses containing a single-stranded RNA molecule as the genome. Following infection, the viral genome is reverse transcribed into double-stranded DNA, which integrates into the host genome and is expressed as proteins. Viral infection using retrovirus requires that target cells must be in their proliferative phase. This constraint may be overcome by using lentiviruses, a subclass of the retroviruses that are able to infect both proliferating and nonproliferating cells, although they are far more complicated to use than retroviruses. Adeno-associated viruses are nonpathogenic human parvoviruses, which depend on a helper virus, usually adenovirus, to proliferate. They are capable of infecting both dividing and nondividing cells, and in the absence of a helper virus integrate into a specific point of the host genome (19q 13-qter) at a high frequency (1).

HSV-1 is a human neurotropic virus that offers the advantage of allowing gene transfer into the nervous system. After infecting neurones, the wild-type HSV-1 virus can either proceed into a lytic life cycle or persist as an intranuclear episome in a latent state. Latently infected neurones function normally and are not rejected by the immune system. Though the latent virus is transcriptionally almost silent, it does possess neurone-specific promoters that are capable of functioning during latency.

This chapter focuses on adenoviruses. Adenoviruses are non-enveloped viruses containing a linear double-stranded DNA genome (Fig. 1). Among over 40 serotype strains of adenovirus, most of which cause benign respiratory tract infections in humans, subgroup C serotypes 2 or 5 are predominantly used as vectors. They are capable of infecting both dividing and nondividing cells. The life cycle does not normally involve integration into the host genome, rather they replicate as episomal elements in the nucleus of the host cell, and consequently there is no risk of insertional mutagenesis. Up to 30 kb out of the 35 kb of the wild-type adenovirus genome can be replaced by foreign DNA (2). There are four early transcriptional units (E1, E2, E3, and E4), having regulatory functions, and a late transcript, which encodes for structural proteins. Progenitor vectors have either the E1 or

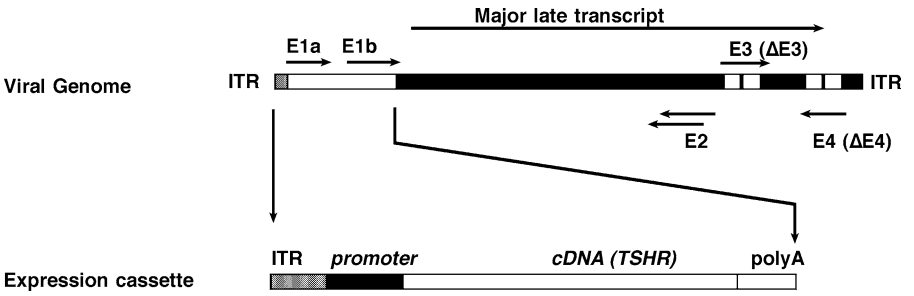

Fig. 1. Schematic representation of the adenoviral genome and of the expression cassette. The sites encoding for the four early transcriptional units (E1, E2, E3, and E4) are indicated. To obtain the recombinant adenovirus, the regions containing the E1a and E1b sites are deleted and the expression cassette is inserted. The expression cassette contains the cDNA of interest (the TSH-R in this case). The deletion of the E1a and E1b cassette prevents the transcription of the major late transcript; this renders the viruses defective for replication and incapable of producing infectious viral particles in target cells. The possible deletion of E3 and/or E4 present in some commercially available viruses is indicated (d E3, d E4). *ITR* inverted terminal repeats.

E3 gene inactivated, and the missing gene can be supplied in trans either by a helper virus, by a plasmid or it could be integrated into a helper cell genome (HEK 293 cells, (3)). Second generation vectors additionally use an E2a temperature-sensitive mutant or an E4 deletion. However, the first generation remains the most widely used (see Note 1).

Viruses with E4 deleted have to be replicated in 911E4 cell lines which can complement E4. The most recent "gutless" vectors contain only the inverted terminal repeats (ITRs) and a packaging sequence around the transgene; all the necessary viral genes being provided in trans by a helper virus (4). In a recent protocol, recombination has been described in prokaryotic cells to improve the yield of recombinant viruses and to facilitate their screening (5, 6). Adenoviral vectors are very efficient in transducing target cells in vitro and in vivo and can be produced at high titers ($>10^{11}$/mL). With only a few exceptions (7), it is generally reported that transgene expression in vivo from progenitor vectors tends to be transient (2).

The essential steps for generating the viral vector are shown in Fig. 2.

Viral infection has a number of useful features:

1. The efficiency of gene transduction is very high (up to 100% in sensitive cells).
2. The infection is easy and does not physically alter the cell membrane for gene transduction.
3. Even cells resistant to transfection with plasmids (including nondividing cells) are susceptible to viral infection.
4. The viral vectors can be used for infection in vivo (including gene therapy) and can potentially be targeted cell-specifically.

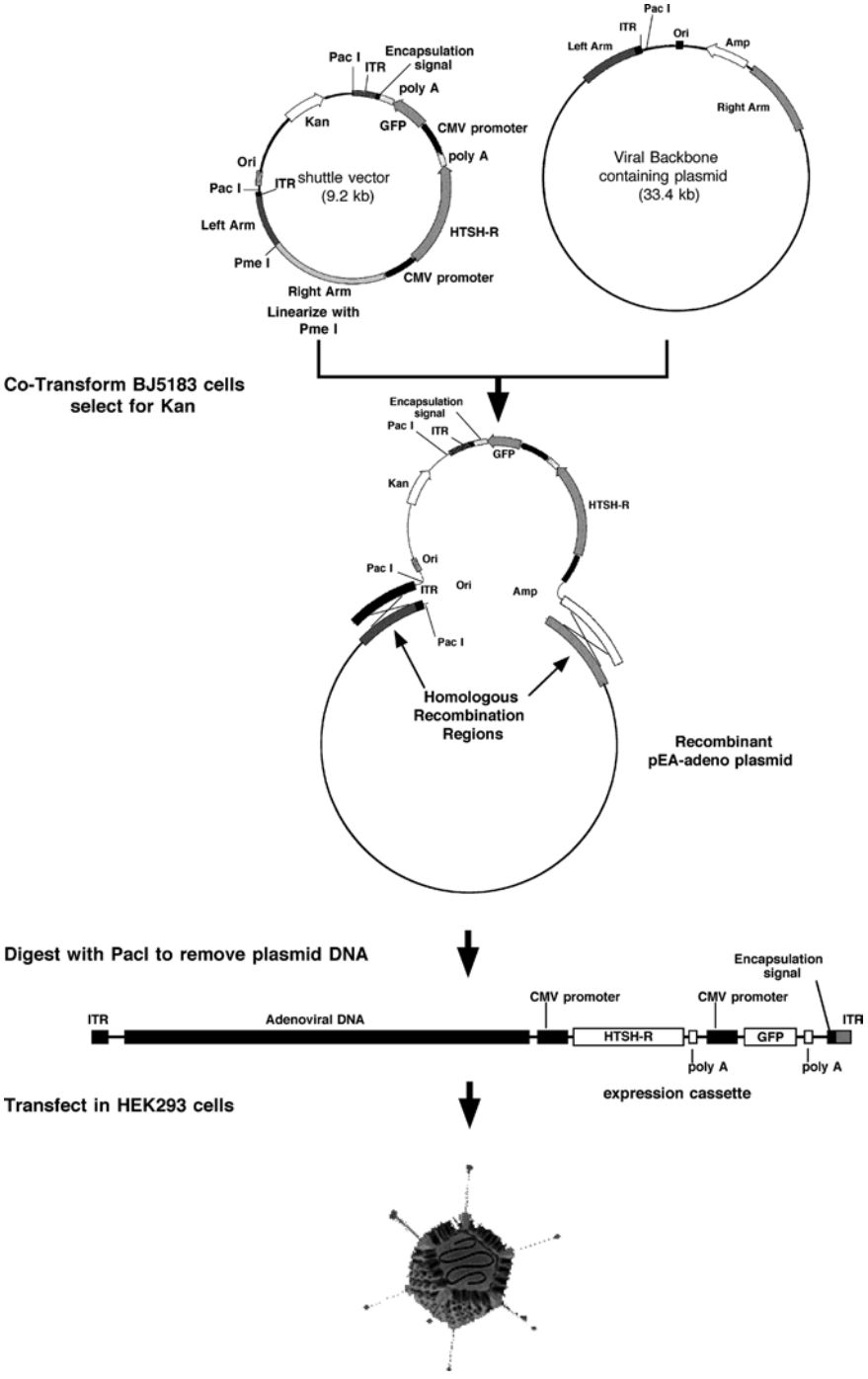

Fig. 2. Schematic representation of the essential steps for generating the recombinant adenovirus. The cDNA of interest (the TSH-R) is cloned into the shuttle plasmid. The viral backbone containing plasmid is composed of the sequence of the adenovirus (Ad5) without the region E1 and E3 (see Fig. 1) plus one sequence derived from the pBR322 containing the *E. coli* ori (origin of replication) and the Amp (ampicillin) resistance. The Bj5183 cells are co-transformed with the linearized shuttle plasmid and the viral backbone containing plasmid for homologous recombination. After recombinant selection, the DNA is digested with PacI, to remove plasmid DNA. The resulting recombinant construct contains the viral backbone plus the expression cassette and is ready for HEK293 transfection. HEK293 provides the transcription factors E1a and E1b for transcription of capsid protein RNAs and viral replication.

The protocol used is a modification of the method described by He and colleagues (5).

The example presented here refers to the thyrotropin receptor (TSH-R) expressed using the pAdTrack-CMV vector.

2. Materials

1. Shuttle vectors: pShuttle-CMV (e.g., Stratagene or Q-Biogene; pAd-TRK-CMV) (see ref. 6).
2. pAdEasy-1 (e.g., Stratagene or Q-biogene).
3. BJ5183 electrocompetent cells.
4. HEK293 cells.
5. Pac I and Pme I restriction enzymes.
6. L Broth: 10 g/L Bacto-triptone, 5 g/L Bacto-yeast, and 5 g/L NaCl.
7. LB-agar: add 15 g/L of bacto-agar to L Broth and autoclave.
8. SOC Medium: 20 g/L Bacto-triptone, 5.5 g/L Bacto-yeast, 10 mM NaCl, and 10 mM KCl; autoclave then add glucose to 20 mM, $MgCl_2$ and $MgSO_4$ to 10 mM each.
9. Electroporation cuvettes, 0.2 cm gap.
10. Lipofectin (Invitrogen).
11. RNAaseA.
12. Neutral Red.
13. Buffer A: 50 mM glucose, 25 mM Tris–HCl pH 8.0, and 10 mM EDTA pH 8.0.
14. Lysis Buffer: 0.2 N NaOH and 1% SDS.
15. Precipitation Solution for 100 mL mix: 5 M potassium acetate 60 mL, glacial acetic acid 11.5 mL, and water 28.5 mL.

3. Methods

3.1. Preparation of Shuttle Plasmid, Adenoviral Backbone Vector, and Competent Cells

1. Subclone the cDNA encoding for the gene of interest (in our case the TSH-R) into the shuttle vector. In this protocol, we refer to the pAdTrack-CMV, which contains the GFP as tracer and the CMV promoter (6) (see Note 2). Prepare the recombinant shuttle vector at high purity (transfection grade) (see Note 3) for the next step. It can be stored at 4°C for up to 6 months.
2. Linearize the recombinant shuttle vector by incubating 1 μg DNA at 37°C for 1 h with the enzyme PmeI (4 units)

in 100 µL reaction buffer (provided by the manufacturer) (see Note 4). After digestion, dilute to 500 µL with TE and extract with 1 volume of phenol/chloroform/isoamidic 25:24:1 followed by ethanol precipitation. For ethanol precipitation, add 1/10 volume of 4 M LiCl, mix and add 2.5 volumes of ice-cold ethanol. LiCl is preferred as it does not interfere with the ligase or with electroporation transfection efficiency. After extraction and purification, resuspend in ultrapure water (15 µL). It can be stored at –20°C. Run 2 µL on an agarose gel to confirm the digestion (see Note 5).

3. The adenoviral backbone vector can be obtained ready-to-use from the manufacturer. This reagent can also be amplified by transforming competent DH5α (as in Subheading 3.2 but using ampicillin and not kanamycin for LB agar) and purified for further experiments (see Note 3).

4. Prepare competent cells (6) as follows: use a fresh colony or frozen stock of DH5α cells to inoculate 10 mL of LB medium in a 50 mL tube (for BJ5183 cells inoculate 10 mL LB containing 30 µg/mL streptomycin). Grow cells in a shaker overnight at 37°C. The day after, dilute 1 mL of cells into 500 mL of LB medium (for BJ5183, use streptomycin-containing LB medium) in a 2 L flask. Grow for 2–4 h with vigorous aeration at 37°C, until A550 is ~0.5 for DH5α and A550 is ~0.7 for BJ5183. Stop cell growth by incubating on ice for 10 min to 1 h (the longer the cells are incubated the higher the competency will be). Collect the cells in two 250 mL conical centrifuge tubes. Pellet cells by centrifuging at 2,600×*g* at 4°C for 10 min. Wash the pellet by resuspending in 500 mL of sterile ice-cold wash buffer (WB; 10% ultra pure glycerol, 90% distilled water v/v). Centrifuge the cell suspension at 2,500×*g* for 30 min. Wash the pellet by resuspending in 250 mL of sterile ice-cold WB. Centrifuge the cell suspension at 2,500×*g* for 15 min. Pour the supernatant off gently leaving about 30 mL. Resuspend and transfer the cell suspension to a 50 mL tube. Spin at 2,500×*g* for 10 min, and pipette all but 5 mL of the supernatant out (for BJ5183 cells, the final total volume should be limited to 2–3 mL). Resuspend the cell pellet in the WB remaining in the tube. Aliquot 20–40 µL per tube (the tubes should be prechilled at –80°C) and store the aliquots at –80°C. You will need at least four aliquots for one recombination. You can store competent cells at –80°C (not in liquid nitrogen). Competent cells are commercially available.

3.2. Generation of Recombinant Adenoviral Plasmids (Fig. 2)

1. To 20 µL competent cells, add 3 µL of linearized shuttle vector and 1 µL (containing 100 ng) of adenoviral backbone vector and mix with the pipette. Transfer into a cuvette (see Note 6) for electroporation. All the reagents, the mix and the cuvette must be on ice.

2. Electroporate (Gene Pulser, Bio-Rad) at 2,500 V, 200 Ω, 25 μF for one pulse.
3. Add to the cuvette 500 μL of prewarmed (37°C) SOC medium, mix, transfer into a fresh tube (15 mL), and grow at 37°C for 20 min.
4. Centrifuge (800 × *g* for 10 min at room temperature), resuspend in 200 μL LB (SOC is fine) and plate in two 10 cm Petri dishes with LB agar with kanamycin 25 μg/mL (see Note 7). Incubate overnight at 37°C.
5. Next day, you should have colonies. Pick up 5–10 colonies (see Note 8) and grow in 3 mL LB plus antibiotic for 15–18 h. Pellet and prepare miniprep as follows: pellet 2 mL overnight culture in 2 mL Eppendorf microfuge tubes, and centrifuge at 15,000 × *g* for 1 min. Discard the supernatants. Add 100 μL Buffer A and vortex briefly. Add 200 μL Lysis Buffer, and gently mix by inverting the tubes several times. Add 150 μL Precipitation Solution, and mix well by inverting the tubes several times. Spin the tubes at 15,000 × *g* for 3 min. Pour the supernatant into fresh 1.5 mL tubes. Add 400 μL of 2-phenol/chloroform (1:1 v/v). Spin at 15,000 × *g* for 5 min (at room temperature). Transfer the upper phase to a new 1.5 mL tube. Add 1 mL ethanol, leave at room temperature for 10 min. Spin at 15,000 × *g* for 5 min (at room temperature). Discard the supernatants. Add 500 μL 70% ethanol, vortex and spin at 15,000 × *g* for 5 min. Discard the supernatants. Briefly spin down (45 s at 15,000 × g) and aspirate the residual liquid in the tubes. Add 30 μL TE/RNAseA (5 μg/mL) to resuspend the DNA. Digest 10 μL of miniprep with PacI (5 units) for 1 h and run on a 0.7% agarose gel. This documents that recombination had occurred (Fig. 3).
6. Take DNA from colonies positive for recombination (as assessed by PacI digestion) and digest for the presence of the insert. For the TSH-R, take 10 μL of recombinant positive colony and digest with BstEII (5 U) for 1 h. Run on a 0.8% gel to assess for the positive band (Fig. 3) (see Note 9).
7. At this stage, you have checked out that the viral backbone has recombined correctly with the plasmid (i.e., it contains the cDNA of interest (the TSH-R) plus the antibiotic resistance), but you do not know whether this construct is able to generate infectious viral particles and to express the protein of interest (the TSH-R) in eukaryotic cells. This is analyzed in Subheading 3.3. For this purpose, transfer (at least) four different insert-positive clones (the 10 μL aliquot remaining after the two digestions) into electro-competent (RecA(−) *Escherichia coli* strains (such as DH5α) (see Notes 8 and 10). Prepare 100–500 μg of transfection-grade purified plasmid (see Note 2) and save stabs from corresponding colonies.

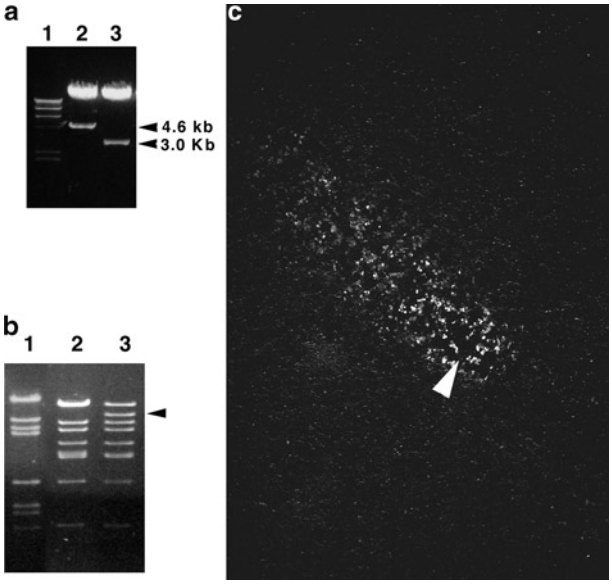

Fig. 3. (**a**) PacI digestion of candidate recombinant clones. This digestion generates two bands: the 33 kb recombinant viral DNA and the 3 kb fragment which contains the kanamycin (Kan) resistance gene. In some clones we found instead a 4.6 kb fragment (likely generated by asymmetric recombination), which turned out to generate infective virus. The presence of a 3–4.6 kb band indicates positive recombination. (**b**) The same clones digested with BstE II to analyze the presence of the insert (TSH-R cDNA). The digestions shown are with the viral backbone containing plasmid (*lane 1*), the recombination without insert (*lane 2*) and the positive clone (*lane 3*). The 9.78 kb band containing the TSH-R cDNA is indicated by the *arrow*. (**c**) Adenovirus-generated foci in HEK293 cells. 7–10 days after transfection the focus is evident and the cells express the GFP tracer. Note that at this stage you cannot see the focus unless you have a tracer (such as GFP).

3.3. Virus Production in Eukaryotic Cells (see Note 11)

1. 24 h before transfection, plate HEK293 cells (5×10^6/T75 flask). Cell should be 50–70% confluent at the transfection.
2. Digest 50 μg of transfection-grade purified plasmid with PacI (100 U) in 250 μL final volume. Run 5 μL on an agarose gel to verify the digestion. Extract and precipitate DNA. Ethanol must be removed in a sterile hood. Resuspend in 100 μL sterile ultrapure water.
3. Transfect HEK293 cells (see Note 12) with digested DNA using Lipofectin. For each flask, prepare 20 μL of PacI-digested plasmid in 1.25 mL of Optimem and 25 μL Lipofectin in 1.25 mL Optimem and leave for 15–40 min at room temperature. Mix these two solutions gently and leave for at least 10 min (is stable for 30–40 min). Wash the HEK293 cells with serum-free medium at least four times, add 3.5 mL Optimem and equilibrate for 15 min at 37°C in a 5% CO_2 incubator. Add the mix to the flask containing Optimem and leave in the incubator for 4 h.

4. Remove the transfection medium and add 10 mL of complete medium with serum. Change the medium every other day.
5. 7–10 days after transfection scrape the cells into the medium and pellet the cells for virus extraction. At this time, unless you have a tracer protein (GFP), you will not usually see clear lysis plaques. The absence of clear plaques at this time does not indicate the absence of recombinant virus (see Note 13) (Fig. 3).
6. Wash the pellet twice in PBS and resuspend in 2 mL PBS and transfer into 1.5 or 2 mL tubes. Freeze and thaw three times in dry ice/ethanol (see Note 14). For thawing, place the frozen tube at 37°C until it starts to thaw and then vortex immediately. Avoid complete thawing at 37°C.
7. Spin at 12,000 × *g* for 30 min and collect the supernatant.
8. Use 1 mL of the supernatant (primary lysate) for the amplification. Save the other 1 mL aliquots at −80°C for the next amplification (stable for up to 6 months).

3.4. Preparation of High Titer Viral Stocks

1. Grow HEK293 cells in flasks to more than complete confluence (better to let them grow 3–4 days after confluence).
2. Wash the cells gently with prewarmed serum-free medium.
3. Add 1mL primary lysate to the cells and gently rock for 2 h at 37°C. Make sure that all the flask surface is in contact with the primary lysate.
4. Remove the primary lysate and add 10 mL medium with serum. Leave cells at 37°C for 72 h.
5. Wash the pellet twice in PBS, resuspend in 2 mL PBS, and transfer into 1.5 or 2 mL tubes. Freeze and thaw three times in dry ice/ethanol (see Note 14). For thawing, place the frozen tube at 37°C until it starts to thaw and then vortex immediately. Avoid complete thawing at 37°C.
6. Spin at 12,000 × *g* for 30 min and collect the supernatant.
7. Titrate the virus in the lysate (see Note 15). The expected titer is $>10^7$PFU/mL.
8. If the viral titer is as expected (see Note 16), infect 5 (or more) T75 flasks of HEK293 cells using this material. Use 2 mL PBS containing 5–10 PFU/cell and proceed as in Subheading 3.4, step 3–6.
9. After centrifugation, you should have 8–10 mL lysate (about 2 mL used for freeze and thaw from five flasks). The expected titer is $>10^9$ PFU/mL. This preparation can be used to infect cells. If you need to further purify the virus suspension or if you need a higher titer (for example to infect in vivo experimental animals), proceed to cesium purification (next step).

10. Add 4.5 g cesium chloride to 8.5 mL of lysate and mix. Transfer this solution to an ultrafuge tube and spin at 150,000 × *g* (SW 41 rotor) for 18–20 h at 10–12°C.
11. Use a needle to collect the viral fraction (within the cesium chloride gradient). Dilute the collected material 1:2 with storage buffer (expected total volume is 1–2 mL), make 200 µL aliquots and store at –20°C (stable for years).
12. Titrate either by plaque assay, by GFP fluorescence determination or by OD determination of viral DNA.

3.5. Virus Titration

1. Remove all but 2 mL per well of medium from six-well plates containing 80–90% confluent HEK293 cells. Infect with appropriately diluted virus (1 mL) for 2 h.
2. Infect cells with 6 different dilution titers (e.g., 10^{-3} to 10^{-8}).
3. Prepare the overlay agar as follows: autoclave 100 mL of 2.8% Bacto-Agar (Difco) and keep warm in a 45°C water bath. To 36 mL of 2.8% Bacto-Agar, add 50 mL of prewarmed 2× BME (GIBCO), 10 mL FBS, 1.25 mL of 1 M $MgCl_2$, and 2 mL of 1 M HEPES. Mix well and swirl at 37°C in a water bath.
4. Add 4 mL/well for a six-well plate. Leave plates at room temperature for 30 min to 1 h.
5. Return the plates to a 37°C, 5% CO_2 incubator.
6. On days 5–7, overlay 2–3 mL agar containing neutral red (from 100× stock, available from GIBCO-BRL) to each well. Plaques should be visible 16–30 h after the neutral red overlay.

3.6. Infection of Target Cells

1. The efficiency of infection and protein expression depends on the target cell type. You should perform preliminary experiments to determine the optimal Multiplicity of Infection (MOI = ratio between PFU and number of cells) and infection procedure.
2. For NIH3T3 cells, subconfluent cells must be covered with the minimal volume (i.e., 1.8–2 mL for a 100 mm dish) of serum-free medium (see Note 17) containing the virus at the MOI of 100 PFU/cell (see Note 18). This MOI is referred to the minimal volume of virus-containing medium on subconfluent cells.
3. After 2 h incubation at 37°C (possibly with rocking), remove the virus-containing medium and add the medium with serum. If needed, the cells can be harvested and plated by 24 h after infection.
4. The protein (TSH-R) is functionally expressed on the cell surface 36–48 h after infection and cells can be used for experiments.

4. Notes

1. A number of companies can prepare viral constructs for infections on demand. For example: NitAn Biothech LLC, 100 Science Village, 1381 Kinnear Road, Columbus provides custom construction services using adenovirus, lentivirus, and adeno-associated viruses (http://www.nitanbiotech.com/viralvectors.php); Q-Biogene (Merlin) provides a custom construction service using adenovirus available for Europe (http://www.qbiogene.com/adenovirus/products/custom/); Vector Biolabs, 3701 Market street, Ste 434, Philadelphia provides custom construction service using adenovirus and adeno-associated viruses (http://www.vectorbiolabs.com/vbs/index.html); Applied Viromics, 4160 Technology Drive, D3, Fremont, CA 94538 provides custom construction service using adenovirus and adeno-associated viruses available for the USA (http://www.appliedviromics.com/Products_1.htm). It is likely that other companies (for example, Invitrogen, Agilent Technologies, Stratagene) could provide a similar service, but it is necessary to contact them and to discuss the specific requests.
2. There are different shuttle vectors commercially available. For example, they may contain the GFP or β-Gal reporter gene. They may also have different promoters or be devoid of promoters to allow the cloning of one promoter of interest to direct the expression of the protein.
3. Use commercially available column purification or CsCl banding.
4. The linearization allows the recombination with the viral backbone and avoids the background of kanamycin-resistant colonies generated by the circular plasmid.
5. If the digestion is incomplete (i.e., <95%), you can purify the linearized DNA from the gel. In this case, extreme care must be taken to avoid any agarose (and perchlorate, if used) residual in the final DNA solution; otherwise, this impairs the electroporation transfection efficiency.
6. Use a 2 mm cuvette and not a 5 mm cuvette.
7. We suggest not to use 50 μg/mL kanamycin, since at this concentration the growth of the recombinant adenoviral plasmid is inhibited.
8. Pick the small colonies as the recombinant containing colonies are usually smaller than those of the shuttle plasmid.
9. Given the size of the recombinant adenoviral plasmid (about 40 kb) and the limited number of diagnostic restriction sites, the digestion may not be sufficient to assess the presence

(and the orientation) of the insert. This point should then be addressed by Southern blotting the digestion.

10. Do not use the BJ5183 strain since these cells allow further recombination of the recombinant adenoviral plasmid.
11. Safety rules for the use of adenoviruses can be found in laboratory manuals or on Web sites (for example, www-ehs.ucsd.edu/bio/biobk/bioap10.htm, or www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4s3.htm).
12. Use care when transfecting HEK293 as these cells tend to detach from the flask, particularly as they are superconfluent. Do not expose cells to Lipofectin for >4 h.
13. At this stage, only a few cells that were infected by the virus have been lysed. After the lysis of these cells, the virus is released and infects the surrounding cells. This is the optimal time to collect the cells to extract the virus (i.e., after infection of surrounding cells and before their lysis, usually 7–10 days after transfection). If you have a GFP tracer, you will see green fluorescent (transfected) cells at day 2 and green infected surrounding cells at days 7–10. If there is no tracer, you can use control plates to see the lysis plaques at day 15–20. This control plate is no longer useful for virus collection (since many infected cells are lysed), but does tell you that the clone is able to generate lytic virus.
14. Always use polypropylene tubes.
15. For titration, you should determine the Plaque Forming Units (PFU) by standard methods. Alternatively, if the virus contains GFP tracer, simply infect superconfluent HEK293 cells with various viral dilutions and count green cells after 24 h. This gives the number of infecting particles, which in our hands corresponds to 5–10 times the PFU value. Determination of PFU is less simple but more quantitative.
16. If the titer is lower, check the transfection efficiency (should be >20%) or start from a different clone.
17. The serum must be carefully removed by washing 2–3 times. For cells that grow in multilayers (such as PC12), it can be difficult to wash out the medium. We suggest that these cells are infected in suspension (in polypropylene tubes) as follows: harvest the cells (using trypsin if needed), wash 2–3 times, and resuspend in a minimal volume (10^7 cells/mL) of virus-containing medium. Incubate for 2 h at 37°C, remove the virus, resuspend in medium with serum and plate.
18. For different cell types, the MOI can vary quite substantially. In our hands, to obtain 80–100% of cells expressing the GFP, we have calculated the following MOI for U251 50PFU/cell, PC12 250 PFU/cell, U87MG 5-10 PFU/cell, COS7 50 PFU/cell, T98G 150 PFU/cell, and for HEK293 5 PFU/cell.

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