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DNA Delivery to Cells in Culture

Generation of Adenoviral Libraries for High-Throughput Functional Screening

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1. Introduction

In functional genomics, the use of expression libraries of DNA variants in combination with potent screening techniques is a powerful tool for gene discovery. They allow study of gene and protein function, generation of peptide variants with novel properties, as well as identification of functional short DNA and RNA motifs. In proteomics, generation of large expression libraries of protein variants with random substitutions ("directed evolution") and further screening for novel or improved functions has been commonly used for isolation of proteins with novel characteristics, for improving enzymes, for rapid isolation of antibodies, and for functional protein studies (reviewed in **refs. *1–3***). Most commonly, peptide libraries are expressed and screened in prokaryotic systems. Such systems have the advantage of rapid and simple generation of clones expressing single variants, allow high diversity (up to 10^{11}), and can be combined with phage- or cell-surface display technique ***(2)***. The main disadvantage of bacterial systems is the absence of posttranslational modifications and native folding of many mammalian proteins, leading to limited applications, particularly when enzyme–substrate-, protein–protein, or protein–RNA interactions are to be studied.

Currently, libraries for screening in mammalian cells have been generated using plasmids, retroviral vectors, or Epstein-Barr virus (EBV)-based vectors

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(4,5). Scoring for new functions or phenotypes upon transfection or transduction led to the identification of genetic suppressor elements, genes involved in growth inhibition and apoptosis, and novel oncogenes. However, more ubiquitous use of plasmid- or retrovirus-based libraries is limited mainly by the range of cells that can be used for efficient transfection or transduction.

Over the past two decades, several generations of replication-deficient, recombinant adenoviruses (AdVs) have been generated and commonly used in gene therapy and functional studies (reviewed in Chapter 1, Part I of this volume; ***6***). Thus far, adenoviral vectors have been used for cloning and delivery of single genes. Here we propose the use of AdVs for generating libraries by positive selection of recombinants. When transient expression is sufficient for functional assessment, adenoviral libraries have several advantages over plasmid- or retroviral-based libraries: (1) they are suitable for a rapid functional screening in a broad range of differentiated, dividing, and postmitotic mammalian cells; (2) small stocks of single adenoviral clones with high titers can be easily obtained; and (3) a high level of uniform transient gene expression can be achieved within 24–48 h postinfection, facilitating the screening process. However, the construction of adenoviral libraries without selection based on inhibited growth of nonrecombinants is fairly inefficient. Even if recombinant clones can be selected using reporter genes, i.e., fluorescent proteins or *LacZ*, in a library of several hundred to several thousand clones there will be a large number of parental viruses that have to be eliminated by time-consuming plaque-purification techniques. Thus, an ideal method for construction of adenoviral libraries would ensure that: (1) a very large number of clones are generated following single transfection, and (2) only recombinant viruses are selected. Among the wide variety of methods used for the construction of recombinant AdVs, several allow generation of recombinants without any parental virus background ***(7–14)***. However, the number of viral clones generated is, at best, lower than 50 per μg of viral DNA, which is insufficient for generating libraries with high diversity.

Recently, we have designed a new type of AdV devoid of the viral protease gene (*PS*) ***(15)***. In a wild-type AdV, the viral protease is expressed during the late phase of infection and is essential for production of mature viral particles ***(16)***. Therefore a *PS*-deficient AdV (Ad5-ΔPS) is capable of a single round of replication but cannot form infectious particles unless propagated in a cell line engineered to express *PS*. We explored this feature to establish a positive selection method based on ectopic co-expression of the *PS* and a gene of interest in the E1 region upon recombination with the Ad5-ΔPS parental genome ***(17)***. With this method, the parental virus can be eliminated after one round of purification because only the recombinant AdVs that have rescued *PS* can lead to productive infection. Furthermore, we applied the positive selection for rapid

generation of adenoviral libraries, as illustrated in **Fig. 1**. The DNA inserts are initially cloned in an Ad5 transfer vector designed to co-express *PS* together with the transgene from two unrelated promotors (**Fig. 1**). The choice of a weak enhancerless promoter for *PS* expression is based on the contention that a low level of *PS* is both sufficient and desirable, and also to minimize promoter interference between the expression cassettes. At the genetic level, the diversity can be generated using a broad range of well-established techniques such as error-prone polymerase chain reaction (PCR), DNA shuffling, random deletions, or cloning cDNAs from various libraries using standard protocols. The method of choice will depend on the particular application and will not be discussed in this chapter.

Because the expression of the gene of interest is not necessary, and could be potentially deleterious during the recombinant AdV generation and production, the various inserts in the library are expressed under the control of a regulated promoter such as the tetracycline inducible (tTA-responsive) promoter ***(18–20)***. Using this system, the recombinant clones are generated and propagated in 293 cells, whereas the functional screening is performed in a cell line expressing a tetracycline-regulated promotor trans-activator (tTA) or by co-infection with a recombinant virus expressing tTA ***(19)***. Alternatively, a strong constitutive promoter such as CMV5 can be used in a configuration, ensuring its repression in specific cell lines expressing a repressor binding to its cognate operator sequence downstream of the start site. We have recently constructed such an inducible system using the *cymene* operon of *Pseudomonas putida* F1 in which the expression of the genes is regulated by a 28kD repressor molecule (CymR) that binds operator sequences downstream of the start site. CymR is in a DNA-binding configuration only in the absence of cymene or cumate, the effector molecules. Thus, when the cumate operator sequences (CuO) was cloned downstream of the start site of the CMV5 promoter, the expression of the transgene was substantially repressed in 293-CymR cells in absence the of cumate ***(21)***. This inducible system permits the construction of AdVs in a cell line in which the transgene expression is minimal (293-CymR), whereas testing of the transgene function can be done by simple transduction of a wide variety of cell lines and primary cells.

The plasmid library is introduced to the cells by transfection following infection with Ad5-ΔPS. Individual clones from the resulting pooled viral stock are isolated as single viral plaques. They are further amplified in small volume stocks and are ready to be used in screening assays. This method allows generation of large adenoviral libraries of several hundred to several thousand clones. We also give an example for high-throughput screening of the amplified adenoviral clones. The screening is designed to determine cell growth and/or viability and is suitable for identification of clones expressing inserts with either cell-

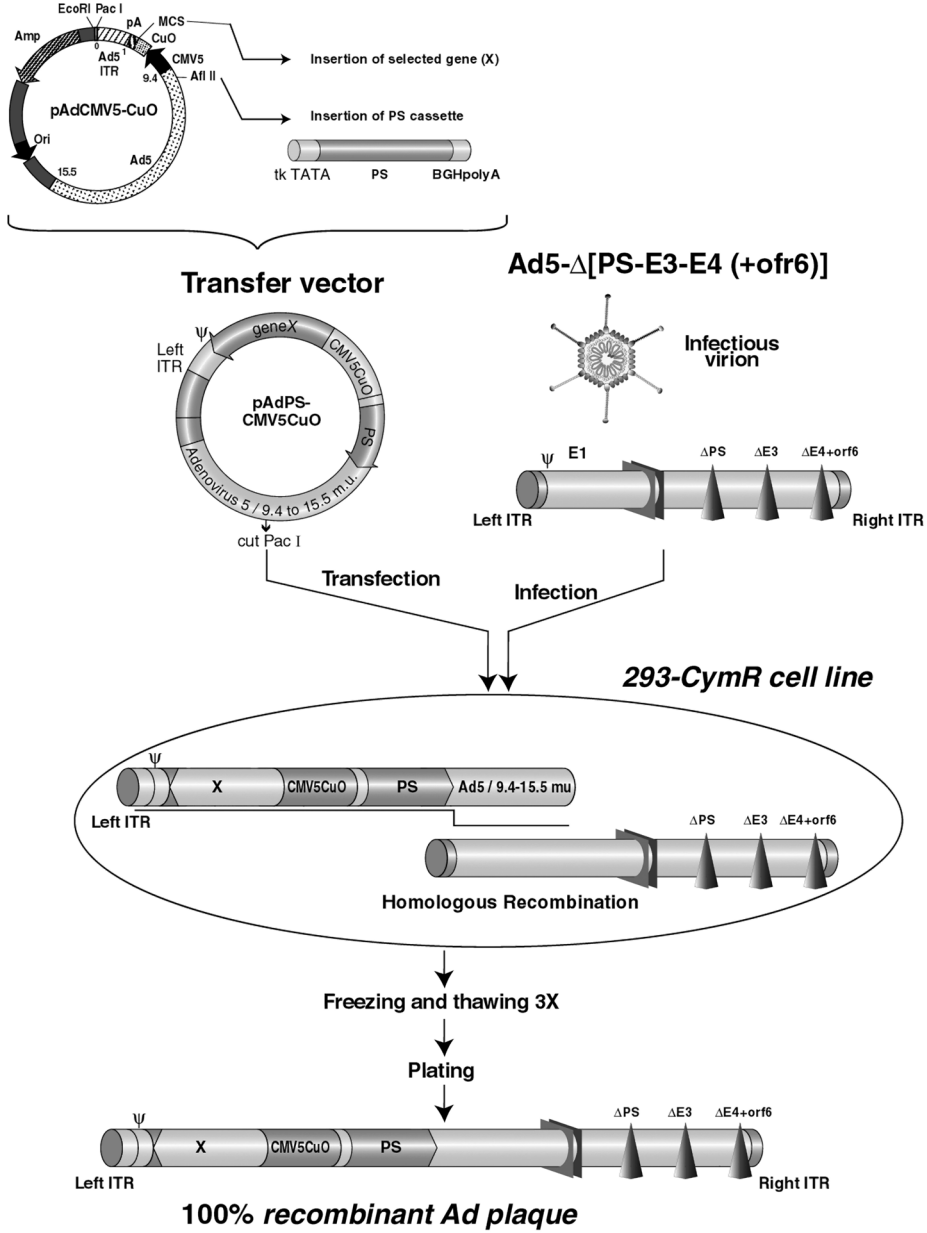

Fig. 1. Schematic representation of the steps required to generate adenoviral library by positive selection of recombinants. The adenoviral transfer vector pAdPS-CMV5CuO used for cloning of DNA variants in the MCS (*Bgl*II/*Not*I/*Eco*RV) carries the left adenoviral ITR and 9.4–15.5 map unit segment of the Ad5 genome for homologous recombi-

cycle dysregulation or pro- or anti-apoptotic properties. Because adenoviral genes in E1, E3, and E4 regions have been shown to interfere with cell-cycle regulation and/or apotosis, the AdVs used for the functional studies are preferably deleted of all of these genes except the E4 orf6. The E1 genes are complemented in 293 cells; the E4 orf6 alone is sufficient for normal growth of E4-deleted virus; and E3 is dispensable for growth in cell culture (reviewed in **ref. *6***). Such AdVs can thus be readily grown in 293-derived cell lines. For functional studies, nonpermissive cells are infected with individual library clones followed by delayed infection with a virus expressing the green fluorescent protein (GFP). Quantification of the resulting GFP expression for each of the co-infections is used as an indication for cell growth or viability. This protocol can include an additional step of apoptosis induction if needed. The assay is performed in 96-well plates and GFP intensity is measured using a FluorImager™ instrument and ImageQuant™ software. A similar assay using GFP as a reporter gene can also readily be developed, for example, to map promoter elements or screen for optimal gene expression in specific cell lines using chimeric promoters assembled with various TATA boxes and enhancer motifs. For other applications, specific assays can further be developed for screening of desired phenotypes.

2. Materials

1. The plasmid pAdPS-CMV5CuO (**Fig. 1**) used to generate libraries is a modified version of pAdCMV5 ***(19).*** In addition to the viral left ITR and an Ad5 region allowing homologous recombination in E1 region, the plasmid contains an expression cassette in which the gene of interest (X) is under the control of a cumate-regulated promoter ***(21)*** and a PS cassette under the control of a tk TATA minimal promotor.
2. Ad5-Δ[PS-E3-E4(+orf6)]: adenovirus type 5 deleted in the *PS* gene as well as in the E3 and E4 regions, except E4 orf6, derived from Ad5-ΔPS ***(15)***, propagated in 293-PS cell line and used as nonpurified viral stock.
3. AdCMV5-GFP ***(20)***: recombinant adenovirus type 5 with deleted E1 and E3 regions ectopically expressing GFP in the E1 region propagated in 293 cells.

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nation with the backbone virus Ad5-Δ[PS-E3-E4(+orf6)]. This plasmid carries two cassettes for ectopic co-expression in the E1 region upon recombination: (1) a cumate-inducible cassette for expression of the gene of interest, and (2) a protease cassette controlled by a tk minimal promotor. Recombinant viruses are generated in 293 cells upon infection with Ad5-Δ[PS-E3-E4(+orf6)] providing the backbone ΔPS viral genome and transfection with linearized transfer vectors. The resulting Δ(E1, E3, E4+orf6) recombinants have rescued PS in the E1 region and can replicate in E1-complementing 293 cells, in contrast to the parental virus.

4. 293 (CRL 1573, ATCC, Manassas, VA), HeLa , and the 293-derived cell line, 293-PS, expressing viral PS ***(15)*** and 293-CymR, expressing the repressor of the cymene operon ***(21)***, are all cultured in Dulbecco`s modified Eagle's medium (DMEM) supplemented with heat-inactivated fetal bovine serum (FBS, HyClone, Logan, UT), 2 m*M* L-glutamine (Wisent Inc., St-Bruno, QC, Canada), 1% antibiotic/antimycotic solution (Wisent Inc.). Use 5% FBS for 293 cells and 10% FBS for all other cell lines. Prepare heat-inactivated FBS by heating the serum at 56°C for 30 min to inactivate complement. For all cells lines, cell growth is at 37°C in a humidified incubator with 5% CO_2 (referred to as standard conditions in the text).
5. Phosphate-buffered saline (PBS) (Wisent Inc.).
6. Trypsin-ethylenediaminetetraacetic acid (EDTA) solution (Wisent Inc.).
7. Stock of 5% Seaplaque GTG agarose (BMA, Rockland, ME). Add 5 g of Seaplaque GTG agarose per 100 mL of PBS. Autoclave for 25–30 min to sterilize. Prepare 10-mL aliquots and store at 4°C. Approximately 1 h prior to preparation of overlay, melt the agarose, add warm complete DMEM to 1% final concentration of Seaplaque agarose, and keep at 42°C until use.
8. Luria-Bertani (LB) medium and LB agar plates prepared according to standard protocols ***(22)***: 10 g of tryptose phosphate, 5 g of yeast extract, 5 g of NaCl, and 15 g of agar for LB agar per l L water; sterilize by autoclaving for 20 min.
9. Twenty-five kDa linear polyethylenimine (PEI) polymer, obtained from Polysciences (Warrington, PA) and prepared according to protocols ***(23)***: stock solution (1 mg/mL) prepared in water and neutralized with HCl. Sterilize the stock solution by filtration using 0.22-µm filter and prepare aliquots of 1 mL. Store at −80°C.
10. QIAGEN Plasmid Maxi Kit (Qiagen Inc., Valencia, CA).
11. TOP10 One Shot™ Chemically Competent Cells (Invitrogen Inc., Carlsbad, CA).
12. *Pac* I restriction endonuclease (New England BioLabs Inc., Beverly, MA).
13. Ampicillin (Sigma, St. Louis, MO): Prepare 100 mg/mL stock in water, aliquot 1 mL samples, and store at −20°C.
14. FluorImager™ instrument and ImageQuant™ software (Molecular Dynamics, Sunnyvale, CA).

3. Methods

3.1. Generation of Ad Library Using Positive Selection

3.1.1. Preparation of Plasmid Library for Transfection

1. Perform cloning of the DNA variants in the multiple cloning site of the adenoviral transfer vector (pAdPS-CMV5CuO, *Bgl*II/*Not*I/*EcoR*V) followed

by transformation of TOP10 One Shot™ Chemically Competent Cells following the manfacturer's instructions.
2. Inoculate the transformation mixture into 250 mL of LB containing 100 µg/mL of ampicillin and grow overnight at 37°C shaking at 300 rpm (*see* **Note 1**).
3. Extract the plasmid DNA using a QIAGEN Plasmid Maxi Kit according to the manufacturer's instructions.
4. Linearize 5–6 µg (per 60-mm dish) of the plasmid variants with *Pac*I , which cuts at the 5′ end of the left ITR (**Fig. 1**), by adding the 2 U of *Pac*I/µg of DNA in total volume of 50 µL of water containing 5 µL of NEB buffer 1 and 100 µg/mL of bovine serum albumin (BSA) (*see* **Note 2**). After overnight digestion, purify the linearized plasmids using standard phenol/chloroform/isoamyl alcohol protocol ***(22)***:
   a. Add 150 µL of water to 50 µL of *Pac*I digested DNA.
   b. Add an equal volume (200 µL) of phenol/chloroform/isoamyl alcohol (25:24:1), and mix by vortexing.
   c. Centrifuge for 1 min at room temperature. Transfer the top (aqueous) phase to a new microcentrifuge tube.
   d. Add 200 µL of chloroform and mix by vortexing.
   e. Repeat **step 4c**.
   f. Add 2 volumes of 100% of ethanol and 1/10 volume of 3 *M* sodium actate, pH 5.2, to the DNA solution, vortex, and incubate at −70°C for 20 min.
   g. Centrifuge for 10 min and wash the pellet with 400 µL of 70% ethanol.
   h. Dry the pellet briefly and resuspend in 20 µL of sterile TE buffer.

3.1.2. Infection/Transfection Protocol for Generating Adenoviral Library

1. Plate 293-CymR cells in 60-mm tissue-culture dishes at 5×10^5 cells per dish (*see* **Note 3**). Incubate overnight at standard conditions.
2. Remove the medium and infect the cells with 1 mL of fresh complete DMEM containing Ad5-Δ[PS-E3-E4(+orf6)] at multiplicities of infection (MOI) of 1–5 plaque forming units (PFUs).
3. Incubate the plates under standard conditions on a rocking platform for 5 h.
4. Replace the infectious mixture with 3 mL of fresh complete DMEM and let the cells recover for 30 min at standard conditions.
5. For each dish, prepare transfection mixture as follows:
   a. To a 1.5-mL microcentrifuge tube add 300 µL of DMEM without serum, 4.5 µg of linearized plasmid library (from **Subheading 3.1.1**.), and 6 µg of PEI ***(22).***
   b. Mix well and incubate for 10 min at room temperature.
6. Add the transfection mixture to the Ad5-Δ[PS-E3-E4(+orf6)] infected cells.

7. Incubate the cells at standard conditions for 3–4 d.
8. Collect the cells together with the medium and subject them to three freeze/thaw cycles to release the viral particles from the cells. The resulting viral mini-stock represents the initial adenoviral library (*see* **Note 4**). Store the mini-stock at −20 to −80°C.

3.1.3. Determination of the Approximate Viral Titer of the Library by Plaque Assay

1. One day prior to plaque assay, plate 293-CymR cells at 2.5×10^5 cells/well into six wells of a 6-well tissue-culture dish.
2. On the day of the plaque assay, make 1:10 serial dilutions (10^{-1} to 10^{-6}) of the viral library mini-stock, each dilution in 1 mL of complete DMEM.
3. Remove the medium from the cells and infect with 1 mL of the appropriate viral dilution per well.
4. Incubate the plates under standard conditions on a rocking platform overnight.
5. Remove the infectious medium and cover the cells in each well with an overlay of 2.5 mL of complete DMEM containing melted Seaplaque agarose at a final concentration of 1%. Leave the dishes at room temperature on a level surface for 5–15 min in order to allow the overlay to solidify.
6. Incubate the dishes under standard conditions for 1 wk. At that time, add a second overlay of 1 mL of fresh DMEM containing 1% Seaplaque agarose to each well.
7. Two weeks postinfection, count the number of plaques per well and multiply by the dilution factor to obtain the approximate virus titer (PFU/mL). Use wells with 10–100 PFUs to estimate the titer.

3.1.4. Plating the Library for Harvesting Individual Viral Clones

1. Plate 293-CymR cells at 1.5×10^6 cells/100-mm tissue-culture dish. The number of dishes depends on the expected library diversity (*see* **Note 3**). Incubate at standard conditions overnight.
2. For each plate, prepare 3 mL of the original viral mini-stock at 50 PFU/mL diluted in complete DMEM.
3. Remove the medium from the cells and infect with 3 mL of the diluted viral mini-stock.
4. Incubate the plates under standard conditions on a rocking platform overnight.
5. Remove the infectious mixture and cover the cells in each dish with an overlay of 10 mL of complete DMEM containing melted Seaplaque agarose at a final concentration of 1%. Leave the dishes at room temperature on a level surface for 5–15 min in order to allow the overlay to solidify.

6. Incubate the dishes under standard conditions for 1 wk. At that time, add a second overlay of 5 mL of fresh DMEM containing 1% Seaplaque agarose to each dish.
7. Viral plaques representing single recombinant clones will appear within 1–2 wk postinfection. At that time, pipet 200 µL of DMEM/well into 96-well plates (*see* **Note 5**).
8. Pick up the viral plaques by piercing the agarose with a 200 µL pipet tip by gently aspirating with a P200 Pipetteman. Transfer the agarose plug to the well and pipet up and down three times. Change the tip after each manipulation.
9. Incubate the plates with the eluted plaques at standard conditions overnight.
10. Store the plate at −20 to −80°C.

3.1.5. Amplification of Individual Viral Clones

1. Plate 293-CymR cells in 96-well plate at 1×10^5 cells per well in 150 µL of complete DMEM. Infect with 50 µL of the eluted plaques.
2. Incubate the plates under standard conditions on a rocking platform for several days until complete cytopathic effect is present in all wells.
3. Subject the plates to three freeze/thaw cycles to release the viral particles from the cells.
4. Store the plate at −20 to −80º C.
5. The amplified viral clones can be further used to infect cells for functional screening assays. At that stage, the titers of the mini-stocks are typically greater than 10^9 PFUs/mL.

3.2. High-Throughput Library Screening for Clones Affecting Cell Viability

The screening assay is designed to measure GFP intensity as an indicator of cell viability. As a basic principle, the infection with individual viral clones is followed by a second delayed infection with AdCMV5-GFP delivering the GFP indicator gene. The assay can be modified for two applications: (1) to identify expressed DNA variants with a pro-apoptotic or cytotoxic phenotype—as a result of reduced cell viability, the synthesis and accumulation of GFP reporter will be impaired; (2) to identify clones with protective or anti-apoptotic properties. In this case, cells can be treated with agents affecting cell viability prior to infection. Increased viability will lead to higher GFP intensity.

1. Inoculate HeLa cells in 96-well plates at 1×10^5 cells per well in 100 µL of complete DMEM (*see* **Note 6**).
2. Infect with 10 µL of the amplified viral clones (*see* **Note 7**) and bring the volume to 200 µL by adding 90 µL of complete DMEM.

3. Incubate the plates under standard conditions on a rocking platform for 12–16 h.
4. Infect with AdCMV5-GFP at MOI of 100 PFU by diluting the viral stock to 1×10^9 PFU/mL and adding 10 µL per well of cells (*see* **Note 8**).
5. Scan the plate on a FluorImager™ for background fluorescence (*see* **Note 9**). Incubate the plate under standard conditions on a rocking platform for 24 h.
6. Scan the plate on a FluorImager to quantify the fluorescence signal. If necessary, incubate the cells for another 24 h and scan again.

3.3. Plaque Purification of Positive Viral Clones

This step is necessary if the positive clones are intended for further applications such as protein production, precise functional studies, or gene therapy. This step assures that the viral clones are homogenous before they are amplified on large scale. One additional round of purification will completely eliminate any possibility of contamination with parental virus or the presence of more than one clone in the preparation.

1. Plate 293-CymR cells at 2.5×10^5 cells per well in 6-well tissue-culture dishes and incubate under standard conditions overnight. Prepare one dish per clone to be purified.
2. The following day, prepare 1:10 serial dilutions (10^{-1} to 10^{-6}) of each eluted plaque (from **Subheading 3.1.4.**) in complete DMEM (*see* **Note 10**).
3. Remove the medium from the cells and infect with 1 mL of virus dilution per well.
4. Incubate the plates under standard conditions on a rocking platform for 2–4 h.
5. Remove the infectious mixture and cover the cells in each well with an overlay of 2.5 mL of complete DMEM containing melted Seaplaque agarose at a final concentration of 1%. Leave the dishes at room temperature on a level surface for 5–15 min in order to allow the overlay to solidify.
6. Incubate the dishes under standard conditions for 1 wk. At that time, add a second overlay of 1 mL of fresh DMEM containing 1% Seaplaque agarose to each well.
7. Two weeks postinfection, collect isolated positive plaques as described in **Subheading 3.1.4., steps 8–10**.

4. Notes

1. To test cloning and transformation efficiency, plate 50 µL of the transformation mixture on LB agar plates containing 100 µg/mL of ampicillin.

2. Other restriction enzymes with even fewer cutting sites, such as *Sce*I, can be used instead of *Pac*I to ensure representation of every insert in the library.
3. The number of plates prepared for infection depends on three parameters. The first parameter is the number of generated independent viral variants, which is dependent on the recombination efficiency. Under optimal conditions, one recombination event per 10^3 cells is expected. The second parameter is library diversity; for example, if the library contains 1000 different DNA variants and the recombination efficiency is optimal, a minimum of 1×10^6 cells have to be initially infected/transfected to ensure the representation of all of the 1000 independent adenoviral clones. The third parameter is the characteristics of the transgenes expressed in the library. Some transgenes are expected to be moderately cytotoxic, pro-apoptotic, or might interfere with the AdV replication. The corresponding viral clones will have impaired growth and, consequently, they will be less represented in the library unless controlled by tight inducible promotors.
4. Along with the pool of recombinant AdV variants expressing PS, the parental virus (Ad5-ΔPS) is also present in the mini-stock because its growth is maintained by trans-complementation. One round of plaque purification (*see* **Subheading 3.1.4.**) is sufficient to eliminate the parental virus and to obtain pure individual viral clones.
5. Ninety-six well plates are convenient because they are compatible for use with multi-channel pipets. This facilitates the next steps of viral amplification and high-throughput functional screening.
6. If the assay is designed for examining cell cycle dysregulation rather than cytotoxicity or anti-apoptotic activity, then 2×10^4 cells per well should be used instead.
7. For more precise results, this assay can be done in triplicate, infecting with 5 μL, 10 μL, and 20 μL of the amplified clones.
8. Given the high amount of GFP released in crude lysates of AdCMV5-GFP-infected 293 cells, CsCl-purified AdCMV5-GFP ***(24)*** should be used to minimize the background GFP that would otherwise be introduced in the wells with viral inocula from crude lysates.
9. For scanning and quantification of the fluorescence intensity, follow FluorImager™ and ImageQuant™ manufacturer's instructions.
10. In most cases, dilutions of 10^{-3} and 10^{-4} are optimal to obtain a few isolated plaques.

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