

Takara Bio USA

Adeno-X™ Adenoviral System 3 User Manual

Cat. No(s). 631180, 632264, 632265, 632266, 632267, 632268 & 632269
(042221)

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I. Introduction & Protocol Overview

A. Summary

Adeno-X Adenoviral System 3 is the most advanced commercially available adenoviral gene delivery system—providing by far the simplest, fastest, and most efficient method for constructing recombinant adenoviral vectors.

Instead of traditional homologous recombination or direct ligation-based methods, our procedure uses **In-Fusion Cloning technology**, which enables directional cloning of any PCR fragment or multiple fragments directly into the linearized adenoviral vector in a single 15-minute reaction. No shuttle vector or additional treatment of the PCR fragment is required (such as restriction digestion, phosphorylation, or blunt-end polishing). This new method now allows you to introduce any cassette into an E1/E3-deleted replication-incompetent human adenoviral vector in just three days and is no more complex than standard plasmid cloning with In-Fusion.

B. Constructing Recombinant Adenovirus with the Adeno-X System 3

To produce recombinant adenoviral vectors using In-Fusion technology, the In-Fusion enzyme links your PCR-generated sequence of interest with the prelinearized pAdenoX vector DNA efficiently and precisely by recognizing a 15-bp overlap at their respective ends. This 15-bp overlap is engineered into the primers used for amplification of the desired sequence.

When the In-Fusion reaction is complete, it is used to transform competent Stellar™ *E. coli* cells (recommended) and recombinant clones are identified by PCR and/or restriction enzyme digestions. The PacI-linearized recombinant adenoviral DNA is then used to transfect a low-passage Adeno-X 293 cell line in order to rescue recombinant adenovirus, which is harvested several days later. Since the recombinant pAdenoX DNA used for transfecting Adeno-X 293 cells originated from an individual bacterial clone, the rescued recombinant adenovirus does not require additional plaque purification.

C. Available Adeno-X System 3 Formats

Adeno-X Adenoviral System 3 is available in seven formats, including the most advanced tetracycline inducible expression system, constitutive expression systems with or without fluorescent reporters, and universal systems that allow you to clone and express an entire expression cassette of your choice.

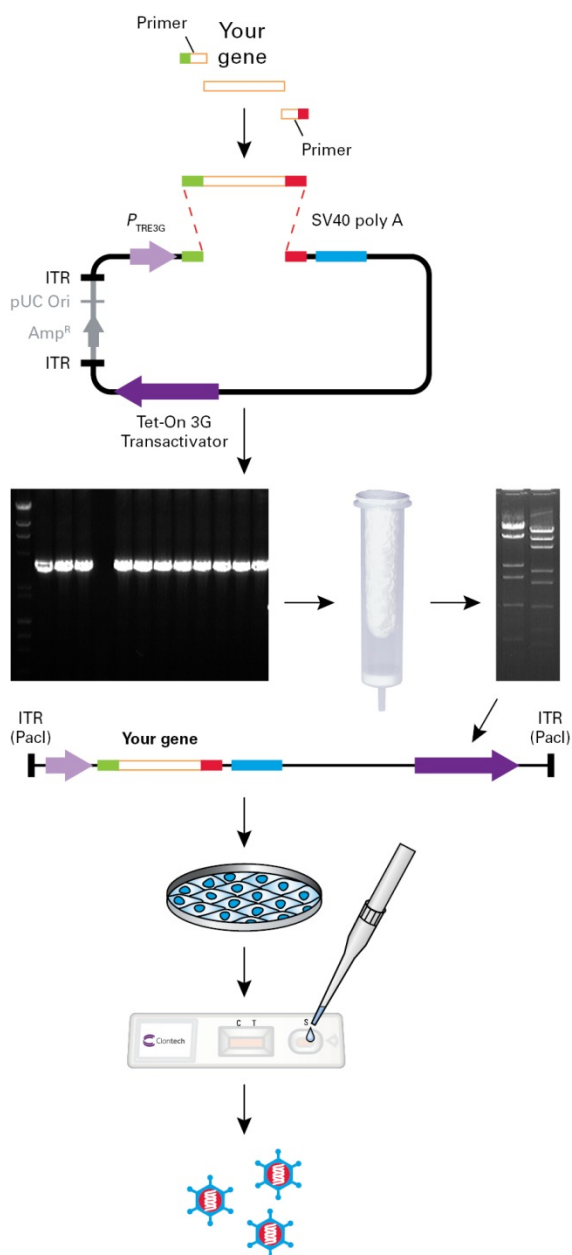
Cat. No.	Product	Description
631180	Adeno-X Adenoviral System 3 (Tet-On 3G Inducible)	- Tightly-controlled, doxycycline-inducible expression system on a single vector - Clone your gene of interest downstream of Takara Bio's <i>P_{TRE3G}</i> promoter, located in the E1 region of the adenoviral genome. - Our highly sensitive Tet-On 3G transactivator cassette is inserted into the E3 region of the adenoviral backbone.

632269	Adeno-X Adenoviral System 3 (CMV)	Constitutive expression of your gene of interest from a CMV promoter
632268	Adeno-X Adenoviral System 3 (CMV, Red)	- Constitutive expression of your gene of interest from a CMV promoter, located in the E1 region of the adenoviral genome. - A DsRed-Express fluorescent protein expression cassette is inserted into the E3 region of the adenoviral backbone. - Easily monitor transduction and virus production.
632267	Adeno-X Adenoviral System 3 (CMV, Green)	- Constitutive expression of your gene of interest from a CMV promoter located in the E1 region of the adenoviral genome. - A ZsGreen1 fluorescent protein expression cassette is inserted into the E3 region of the adenoviral backbone. - Easily monitor transduction and virus production.
632266	Adeno-X Adenoviral System 3 (Universal)	- Use any promoter, gene, and polyA sequence. - Ideal for tissue-specific expression or expression of shRNA or miRNA
632265	Adeno-X Adenoviral System 3 (Universal, Red)	- Use any promoter, gene, and polyA sequence. - Ideal for tissue-specific expression or expression of shRNA or miRNA - A DsRed-Express fluorescent protein expression cassette is inserted into the E3 region of the adenoviral backbone. - Easily monitor transduction and virus production.
632264	Adeno-X Adenoviral System 3 (Universal, Green)	- Use any promoter, gene, and polyA sequence. - Ideal for tissue-specific expression or expression of shRNA or miRNA - A ZsGreen1 fluorescent protein expression cassette is inserted into the E3 region of the adenoviral backbone. - Easily monitor transduction and virus production

D. Replication-Incompetent Adenovirus Provides Added Safety & Control

To accommodate DNA inserts and to produce a replication-incompetent adenoviral vector, extensive portions of the Early Regions 1 (E1) and 3 (E3) of wild-type adenovirus have been deleted from the Ad5 genome in our pAdenoX vectors—enabling you to ligate **up to 8 kb** of foreign DNA into these vectors without adversely affecting the efficiency of viral particle formation (see Table I in Section VII.D for pAdenoX vector capacities). Because the E1 elements have been eliminated, an early passage Adeno-X 293 cell line is required to propagate and titrate recombinant adenoviruses derived from Adeno-X Viral DNA (Graham *et al.*, 1977; Aiello *et al.*, 1979). Adeno-X 293 cells stably express the Ad5 E1 genes that are essential for replication and transcription of Adeno-X Viral DNA. The Adeno-X genome carries the remaining coding information necessary to produce fully functional viral particles. The E3 region of the adenoviral genome is nonessential for adenovirus replication.

In addition to creating room for DNA inserts, E1 region deletion restricts the cytopathic activity of the recombinant adenoviral particles produced by this system (Graham *et al.*, 1977). This valuable safety feature means that when you clone your expression cassette into Adeno-X Viral DNA, you produce an infectious but replication-incompetent adenovirus, which propagates only in those cell types (e.g., Adeno-X 293 cells) that express the E1-encoded trans-complementing factors. For all other somatic cell types susceptible to adenoviral infection, exposure to your recombinant adenovirus leads to a transient non-cytopathic (i.e., non-lytic) infection. The adenoviral genome in infected cells is neither replicated nor actively transcribed, since the cell lacks the necessary transcription factors—the E1 gene products. Although the Ad genes remain inactive, your gene insert is still expressed at high levels because it is independently controlled by an exogenous promoter (e.g., the cytomegalovirus immediate early promoter, *P_{CMV IE}*). Expression of your gene from the Adeno-X genome is independent of target cell proliferation or the presence of any other viral genes or promoters.



1. Amplify your gene with 15 bp of homology to pAdenoX

2. Clone using In-Fusion technology and Stellar Competent Cells

3. Perform colony PCR to verify correct clone(s)

4. Purify pAdenoX-GOI using NucleoBond Xtra

5. Digest with XhoI/NheI to verify integrity

6. Digest with PacI (to linearize)

7. Transfect Adeno-X 293 cells

8. Confirm the presence of adenovirus with Adeno-X GoStix

9. Harvest recombinant adenovirus

Figure 1. Constructing recombinant adenovirus with In-Fusion technology. DNA sequences can be rapidly transferred as PCR products to any pAdenoX vector using the In-Fusion cloning method. In this example, your gene of interest is amplified with 15 bp extensions that are homologous to the ends of the linearized adenoviral vector. The PCR product is then purified and mixed with the linearized adenoviral vector of choice in the In-Fusion reaction. Following the reaction, a portion of the mixture is transformed into *E. coli* (Stellar Competent Cells) and screened. Once a PCR-positive clone is identified, the recombinant pAdenoX vector is amplified, purified, and subsequently linearized with the restriction enzyme PacI, then transfected into Adeno-X 293 cells for viral rescue and amplification. Adeno-X GoStix™ can be used to determine the status of adenovirus rescue.

II. List of Components

For storage conditions, refer to the Certificate of Analysis supplied with each component.

Each kit includes sufficient reagents to perform 10 reactions.

A. Adeno-X Expression System 3

Choose from the following 7 different system formats:

<u>Cat. No.</u>	<u>System Name</u>
631180	Adeno-X Adenoviral System 3 (Tet-On 3G Inducible)
632269	Adeno-X Adenoviral System 3 (CMV)
632268	Adeno-X Adenoviral System 3 (CMV, Red)
632267	Adeno-X Adenoviral System 3 (CMV, Green)
632266	Adeno-X Adenoviral System 3 (Universal)
632265	Adeno-X Adenoviral System 3 (Universal, Red)
632264	Adeno-X Adenoviral System 3 (Universal, Green)

B. General System Components

All systems listed in Section II.A contain the following 7 components:

- 10 µl Linearized pAdenoX Vector DNA (200 ng/µl)
- 50 µl Adeno-X Screening Primer Mix 3 (10 µM)
- 20 µl Adeno-X Control Fragment (50 ng/µl)
- 10 rxns In-Fusion Snap Assembly Master Mix (also sold separately as Cat. No. 638947)
- 10 preps NucleoSpin Gel and PCR Clean-Up Kit (also sold separately as Cat. No. 740609.10)
- 10 rxns Stellar Competent Cells (also sold separately as Cat. No. 636763)
- 10 preps NucleoBond Xtra Midi Kit (also sold separately as Cat. No. 740410.10)

III. Additional Materials Required

The following materials are required but not supplied:

A. pAdenoX DNA Manipulations

- Ampicillin (Amp): Prepare a 50 mg/ml stock solution. Store at –20°C.
- LB liquid and agar media
- Glycogen (20 mg/ml)
- Agarose
- Sterile, deionized H₂O
- 10 M (saturated solution) ammonium acetate (NH₄OAc) or 3 M sodium acetate (NaOAc; pH 5.2)
- Sodium dodecyl sulfate (SDS)
- Restriction endonucleases: PacI, XhoI & NheI (New England Biolabs)
- 1X TE Buffer (10 mM Tris-HCl [pH 8.0]; 1 mM EDTA)
- Phenol:chloroform:isoamyl alcohol (25:24:1): Equilibrate with 100 mM Tris-HCl (pH 8.0)
- Ethanol (100% and 70%)

B. PCR

- CloneAmp™ HiFi PCR Premix (Cat. No. 639298) for In-Fusion Cloning
- Terra™ PCR Direct Red Dye Premix (Cat. No. 639286)

C. Mammalian Cell Culture Supplies

- Adeno-X 293 Cell Line (Cat. No. 632271)
Used to package and propagate the recombinant adenoviral-based vectors produced with the Adeno-X Expression System. The Adeno-X 293 Cell Line may be grown in DMEM. Supplement the medium with 100 units/ml penicillin G sodium, 100 µg/ml streptomycin, 4 mM L-glutamine, and 10% fetal bovine serum.
- Dulbecco's Modified Eagle's Medium (DMEM)
- Solution of 10,000 units/ml penicillin G sodium and 10,000 µg/ml streptomycin sulfate
- Fetal Bovine Serum
- Trypsin-EDTA
- Phosphate-Buffered Saline (PBS, without Ca²⁺ and Mg²⁺)
- Cell Freezing Medium, with or without DMSO (Sigma, Cat. Nos. C6164 or C6039)
- Tissue culture plates and flasks (e.g., 60 mm plates, 6-well plates, T75 & T175 flasks)
- Trypan Blue Dye (0.4%)
- Doxycycline (Cat. No. 631311)

D. Transfection of pAdenoX DNA into Adeno-X 293 Cells

Takara Bio recommends using the calcium phosphate transfection method for transfecting large plasmids into Adeno-X 293 cells.

- CalPhos™ Mammalian Transfection Kit (Cat. No. 631312)

E. Tet-On 3G Inducible Expression (for use with Cat. No. 631180)

- 5 g Doxycycline (Cat. No. 631311)
Dilute to 1 mg/ml in double distilled H₂O. Filter sterilize, aliquot, and store at –20°C in the dark. Use within one year.
- Tetracycline-Free Fetal Bovine Serum
Contaminating tetracyclines, often found in serum, will significantly elevate basal expression when using Tet-On 3G. The following functionally tested tetracycline-free sera are available from Takara Bio:

<u>Cat. No.</u>	<u>Serum Name</u>
631106	Tet System Approved FBS (500 ml)
631107	Tet System Approved FBS (50 ml)
631367	Tet System Approved FBS (3 x 500 ml)
631101	Tet System Approved FBS, US-Sourced (500 ml)
631105	Tet System Approved FBS, US-Sourced (50 ml)
631368	Tet System Approved FBS, US-Sourced (3 x 500 ml)

F. Determination of Adenoviral Titers

- Adeno-X Rapid Titer Kit (Cat. No. 632250)
- Adeno-X qPCR Titration Kit (Cat. No. 632252)
- Adeno-X GoStix (Cat. No. 632270)

G. Purification of Adenovirus

Adenoviral stocks are produced using Adeno-X 293 cells and virus is harvested when the cytopathic effect (CPE) is considered to be complete. Because the virus is harvested from the cell lysate, it is very important to purify the virus away from the cell debris present in the lysate. Contaminating cellular DNA, proteins, and membrane fragments can be cytotoxic to target cells even in small amounts. Therefore, we recommend purifying your adenoviral vectors using one of the kits mentioned below. The Adeno-X Maxi and Mega Purification Kits utilize a chromatography-based approach that provides a rapid, scalable, and high-yield alternative to CsCl-based methods.

<u>Cat. No.</u>	<u>Product</u>
631532	Adeno-X Maxi Purification Kit (2 preps)
631533	Adeno-X Maxi Purification Kit (6 preps)
631032	Adeno-X Mega Purification Kit (2 preps)

IV. Safety & Handling of Adenoviruses

The protocols in this User Manual require the production, handling, and storage of infectious adenovirus. It is imperative to fully understand the potential hazards of and necessary precautions for the laboratory use of adenoviruses. The National Institute of Health and Center for Disease Control have designated adenoviruses as Level 2 biological agents. This distinction requires the maintenance of a Biosafety Level 2 facility for work involving this virus and others like it. The virus packaged by transfecting Adeno-X 293 cells with the adenoviral-based vectors described here are capable of infecting human cells. These viral supernatants could, depending on your gene insert, contain potentially hazardous recombinant virus. Similar vectors have been approved for human gene therapy trials, attesting to their potential ability to express genes *in vivo*. For these reasons, due caution must be exercised in the production and handling of any recombinant adenovirus. **The user is strongly advised not to create adenoviruses capable of expressing known oncogenes.**

For more information on Biosafety Level 2, see the following reference:

- Biosafety in Microbiological and Biomedical Laboratories (BMBL), 5th Edition (December 2009)
U.S. Department of Health and Human Services, CDC, NIH.
(Available at <http://www.cdc.gov/biosafety/publications/bmbl5/index.htm>)

Biosafety Level 2:

The following information is a brief description of Biosafety Level 2. *It is neither detailed nor complete.* Details of the practices, safety equipment, and facilities that combine to produce a Biosafety Level 2 are available in the above publication. If possible, observe and learn the practices described below from someone who has experience working with adenoviruses.

A. Practices

- Perform work in a limited access area
- Post biohazard warning signs
- Minimize aerosols
- Decontaminate potentially infectious wastes before disposal
- Take precautions with sharps (e.g., syringes, blades)

B. Safety Equipment

- Biological safety cabinet, preferably Class II (i.e., a laminar flow hood with microfilter [HEPA filter] that prevents release of aerosols; not a standard tissue culture hood)
- Protective laboratory coats, face protection, double gloves

C. Facilities

- Autoclave for decontamination of waste
- Unrecirculated exhaust air
- Chemical disinfectants available for spills

V. General Considerations: Preparing Recombinant pAdenoX Adenoviral DNA

When maintained in bacterial culture for long periods, adenoviral plasmid DNA can become resistant to restriction digestion, or produce smaller derivatives that eventually outgrow the original construct.

To avoid such aberrations, always follow these guidelines:

A. Use Stellar Competent Cells

- Adeno-X Adenoviral System 3 has only been validated for use with competent Stellar *E. coli* cells. We cannot guarantee cloning performance or plasmid stability when this system is used with other competent cells.

B. Start with Fresh Cell Cultures

- Start with freshly transformed cells. Do not store frozen glycerol stocks of *E. coli* cells containing adenoviral DNA.
- Always use fresh, log-phase cultures as your source of recombinant pAdenoX plasmid DNA.
- Do not store a culture at room temperature, 4°C, or on ice for long periods (i.e., >24 hr) before starting a purification or inoculating a second culture.

C. Minimize Culture Time

- After plating transformants on LB/Amp plates, pick the resulting colonies as soon as possible (i.e., within 24 hours). Without delay, amplify the clones in 5 ml of LB/Amp liquid broth.

- Meanwhile, perform PCR analysis. If PCR confirms the presence of a recombinant pAdenoX construct, transfer a portion of the corresponding 5 ml, log-phase culture to 100 ml of fresh LB/Amp broth to further amplify the selected clone.
- When this culture reaches log phase, purify the recombinant construct using the NucleoBond Midi-Scale procedure, as described in Section VIII.C. NucleoSpin Kits or other miniprep spin columns should not be used to purify pAdenoX DNA.

D. Purify Adenoviral DNA Using NucleoBond Xtra Midi

We strongly recommend the use of NucleoBond Xtra for all pAdenoX DNA purifications. NucleoBond Xtra is the highest performing gravity-based plasmid purification kit and is ideal for purifying intact large plasmids such as pAdenoX.

- The purified plasmid is high quality and transfection grade
- Large plasmids such as pAdenoX (34–38 kb) are sheared on miniprep spin columns, but Nucleobond Xtra can purify intact plasmids as large as 300 kb
- A NucleoBond Xtra 10 prep kit is supplied with your system, but additional kits are available from Takara Bio

<u>Cat. No.</u>	<u>Product Name</u>	<u>Size</u>
740410.10	NucleoBond Xtra Midi	10 Preps
740410.50	NucleoBond Xtra Midi	50 Preps
740410.100	NucleoBond Xtra Midi	100 Preps

VI. Cell Culture Guidelines

A. General Guidelines for Adeno-X 293 Cells

1. References

We recommend our Adeno-X 293 cell line, a slower-growing, more adherent line used to package and propagate the recombinant adenovirus-based vectors produced with the Adeno-X Adenoviral Expression System 3. For more information on mammalian cell culture, we recommend the following references:

- *Culture of Animal Cells*, Fifth Edition, ed. by R.I. Freshney (2005, Wiley-Liss)
- *Current Protocols in Molecular Biology*, ed. by F.M. Ausubel *et al.* (1995 *et seq.*) John Wiley & Sons, Inc.

2. Growing Cells

- Adeno-X 293 cells should be grown in a monolayer, preferably in plastic tissue culture dishes or flasks. Under optimum growth conditions (37°C, 5% CO₂), 293 cells double about every 36 hr.
- To maintain consistency, do not passage cells indefinitely. For best results, we recommend you use slower growing, early passage Adeno-X 293 cells for transfection and titration procedures.

3. Preventing Contamination

- To prevent contamination, work with media and uninfected cells in a vertical laminar flow hood, using sterile technique. Keep this hood free of virus to prevent accidental infection of the stock cultures; ideally use another hood for all virus work.
- All virus-contaminated materials, including fluids, must be autoclaved or disinfected with 10% bleach or a chemical disinfectant before disposal.

B. Protocol: Maintaining Adeno-X 293 Cells in Culture

1. To thaw Adeno-X 293 cells, place the vial of frozen cells in a 37°C water bath until just thawed. Sterilize the outside of the vial with 70% EtOH. For maximum viability upon plating, remove DMSO as follows:
 - a. Add 1 ml complete medium (prewarmed to 37°C). Transfer mixture to a 15 ml tube.
 - b. Add 5 ml complete medium and mix gently. Repeat. The final volume should be 12 ml.
 - c. Centrifuge at 125 x g for 10 min. Remove supernatant.
 - d. Gently resuspend cells in 10 ml complete medium: DMEM supplemented with 100 units/ml penicillin G sodium, 100 µg/ml streptomycin, 4 mM L-glutamine, and 10% fetal bovine serum.
2. Transfer cells (in 10 ml of growth medium) to a 100 mm culture plate.
3. Cells should be split every 2–4 days when they reach 80–90% confluency.
Do not seed cells too sparsely or allow them to become over confluent.
4. Split the cells as follows:
 - a. Remove the medium and wash the cells once with sterile PBS (containing no Ca²⁺ or Mg²⁺).
 - b. Add 1–2 ml of trypsin-EDTA solution and treat for 1–3 min, just long enough to detach cells (do not expose cells to trypsin for extended periods). Then add 5–10 ml of complete growth medium (to stop trypsinization) and resuspend the cells gently but thoroughly.
 - c. Transfer the desired number of cells to a 100 mm plate containing 10 ml of medium. Gently rock the plate to distribute cells.

C. Protocol: Preparing Frozen Cultures of Adeno-X 293 Cells

We recommend you prepare frozen aliquots of early-passage Adeno-X 293 cells to ensure a renewable source of cells.

1. Expand the cell line in the desired number of flasks or plates.
2. When the desired number of flasks/plates have reached ~80% confluence, wash the cells once with sterile PBS (containing no Ca^{2+} or Mg^{2+}), trypsinize, add 2–4 volumes complete medium to dilute the trypsin, and harvest the cells.
3. Count your cells (using trypan blue exclusion or other method) and collect by centrifugation (~500 x g for 10 min).
4. Resuspend in 4°C Cell Freezing Medium at $1\text{--}2 \times 10^6$ cells/ml.
5. Dispense 1 ml aliquots into labeled cryovials and place in a cell freezing container (reduces temperature ~1°C/min) at –80°C overnight. Alternatively, place the vials on ice or at –20°C for 1–2 hours, transfer to an insulated container (foam ice chest), and place container in a –80°C freezer for several hours to overnight.
6. Transfer vials to liquid nitrogen.
7. Two or more weeks later, confirm the viability of frozen stocks by starting a fresh culture as described in Section VI.B.

VII. In-Fusion® Cloning Procedure for Adenoviral DNA

For more detailed information regarding In-Fusion cloning, please refer to the In-Fusion Snap Assembly User Manual (available at takarabio.com/manuals).

A. PCR Amplification of Insert

For the best results, we recommend using our **CloneAmp HiFi Premix** (Cat. No. 639298), which offers high-fidelity, efficient amplification of long gene segments (>1 kb), and automatic hot start for increased specificity and reduced background.

1. Use the following amounts of template for each 50 µl PCR reaction:
 - Human Genomic DNA: 5 ng–200 ng
 - cDNA: 1 ng–200 ng
 - Plasmid DNA: 10 pg–1 ng
2. Consider the following guidelines when amplifying your gene of interest:
 - For the Tet-On 3G System or any CMV System, in most cases the final amplified sequence will consist of a gene of interest from start to stop codon, flanked by 15 bp of pAdenoX sequence.
 - For the Universal Systems, in most cases the amplified sequence will consist of an entire expression cassette containing a promoter, gene (or shRNA) sequence, and polyA signal, flanked by 15 bp of pAdenoX sequence.
 - It is also possible to construct an expression cassette in any Universal System using multiple fragment cloning in a single reaction (see Appendix B for details).

B. PCR Primer Design

In-Fusion technology allows you to join two or more fragments, e.g., a vector and insert (or multiple fragments), as long as they share 15 bases of homology at each end. Therefore, the PCR primers used to amplify your gene or expression cassette must be designed in such a way that each end of the PCR product generated shares 15 bp of homology with one end of the linearized pAdenoX vector (Figure 2). A specific example is given using the *lacZ* gene.

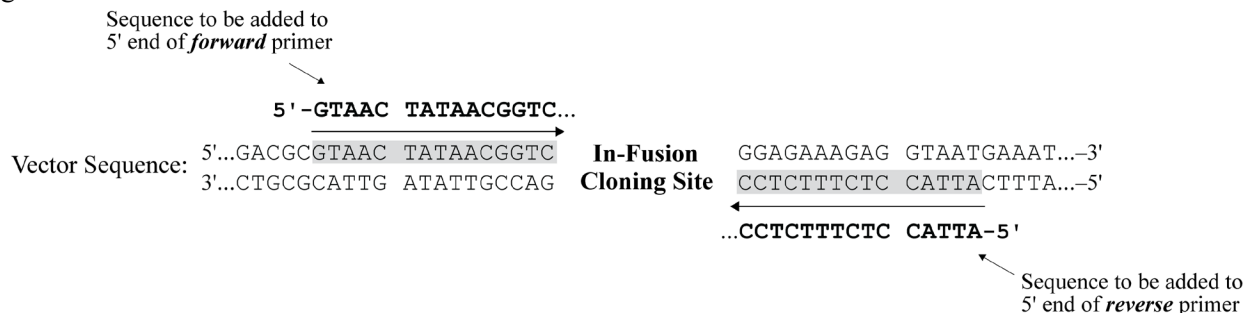


Figure 2. In-Fusion primer design example.

1. General Primer Design Guidelines:

The required forward/reverse primer designs are as follows:

Forward Primer* = gtaactataacggtc 111 222 333 444 555 666 777 888

Reverse Primer** = attacctctttctcc LLL NNN NNN NNN NNN NNN NNN NNN

*111 = first 3 nucleotides of your gene sequence

(This is likely to be the start codon if you are using the Tet-On 3G system or any CMV System.)

*222 = second 3 nucleotides; etc.

**LLL = reverse compliment of the last 3 nucleotides of your sequence (e.g., the stop codon)

**NNN = reverse compliment of the end of your sequence.

Specific Primer Design Example Using the *lacZ* Gene:

Forward Primer = gtaactataacggtc *atg tcg ttg act ttg acc aac aag*

Reverse Primer = attacctctttctcc *tta ttg tga aca cca gac caa c*

Letters in italics are specific for the *lacZ* gene and include a start codon "atg" (at the start of the Forward Primer sequence) and a stop codon "taa" (at the end of the reverse complement of the Reverse Primer sequence). Note these primers were used to amplify the control fragment supplied with your kit.

2. Amplify the sequence that you wish to clone by PCR using CloneAmp HiFi Premix.
3. If you amplified your PCR product from a plasmid containing an ampicillin resistance marker, we recommend digesting your PCR product with DpnI to remove any contaminating DNA template prior to spin column purification.

C. Protocol: Spin Column Purification of PCR Fragments

1. Following PCR amplification, verify your results by analyzing a small portion of your PCR reaction on an agarose gel.
2. Spin column-purify the remainder of your PCR product by **NucleoSpin Gel and PCR Clean-Up** (supplied, see Section II.B). During purification, avoid nuclease contamination and exposure of the DNA to UV light for long periods of time.

NOTE: Although spin column purification results in increased cloning efficiency, you can gel-purify your PCR product if nonspecific background bands are observed in your PCR reaction.

3. After purification, proceed with the protocol for “In-Fusion Cloning of Spin Column-Purified PCR Fragments” (Section VII.D).

D. Protocol: In-Fusion Cloning of Purified PCR Fragments

When using this procedure, the following amounts of spin column-purified insert and vector are recommended for optimum cloning efficiency:

- If the PCR fragment is shorter than 0.5 kb, maximum cloning efficiency may be achieved by using less than 50 ng of fragment.
- The Adeno-X Control Fragment consists of a complete *lacZ* gene flanked by 15 bp of sequence homologous to the ends of pAdenoX. It is designed as a cloning control, but the gene will also be expressed in the Tet-On 3G and CMV Systems so *lacZ* expression can be assayed in these systems.
- To determine the largest PCR fragment that can theoretically be cloned into your linearized pAdenoX vector without adversely affecting viral function, see Table I.

Table 1. Theoretical pAdenoX Vector Capacities

Vector	Cloning Capacity
pAdenoX-Tet3G	4.6 kb
pAdenoX-CMV	6.4 kb
pAdenoX-DsRed-Express	4.8 kb
pAdenoX-ZsGreen1	4.8 kb
pAdenoX-PRLS	8.0 kb
pAdenoX-PRLS-DsRed-Express	6.4 kb
pAdenoX-PRLS-ZsGreen1	6.4 kb

Perform the In-Fusion reaction as follows:

1. Set up the In-Fusion reactions in 0.2 ml PCR tubes. Add the reagents in the order shown below:

Reagent	Reagent Volume (µl per sample)	
	Cloning Reaction	Control Reaction
Deionized H ₂ O	5	5
Linearized pAdenoX vector (200 ng/µl)	1	1
PCR insert (50 ng/µl)	2	0
Adeno-X Control Fragment (50 ng/µl)	0	2
In-Fusion Snap Assembly Master Mix	2	2
Total volume per rxn	10	10

2. Incubate the reactions for **15 min at 50°C**, then place on ice.
3. Proceed with the Transformation Procedure in Section VIII.A. You can store the cloning reactions at –20°C until you are ready.

VIII. Transformation, Screening & Purification of Recombinant Adenoviral Constructs

A. Protocol: Transformation Using Stellar™ Competent Cells

1. Add 1.5 µl of the In-Fusion reaction mixture to 1 tube of Stellar cells (100 µl)
2. Incubate on ice for 30 min.
3. Heat shock for 45 sec at 42°C.
4. Incubate on ice for 2 min.
5. Add 900 µl SOC Medium and shake at 250 rpm at 37°C for 1 hr.
6. Spread 100 µl (~10%) of each transformation onto LB agar plates containing 100 µg/ml ampicillin.
7. Centrifuge the remainder of each transformation reaction at 6,000 rpm for 5 min.
8. Discard the supernatant and resuspend each pellet in 100 µl of fresh SOC medium. Spread each sample on a separate LB ampicillin plate. Incubate all of the plates overnight at 37°C.
9. Pick individual, well-separated colonies from the plates for screening in Section VIII.B.

NOTE: On rare occasions after transformation and plating, a mixture of small and large colonies may be observed. In this case, pick the smallest colonies for screening for the recombinant adenoviral vector.

B. Protocol: PCR Colony Screening of Clones Using the Terra™ PCR Kit

Use the following method to screen your clones for the presence of an insert using the Adeno-X Screening Primer Mix 3.

1. Using a sterile toothpick or pipette tip, transfer a single, randomly chosen colony into 40 µl of deionized H₂O. We typically screen about 5–8 colonies.
2. Resuspend the colony by gently vortexing or pipetting up and down.
3. From the 40 µl suspension, do the following:
 - a. Transfer 20 µl of the suspension into 5 ml of liquid LB Medium containing 100 µg/ml ampicillin (LB/Amp). Incubate at 37°C with shaking for 6–8 hr. This will be used for later amplification of positive colonies.
 - b. Analyze 5 µl of each clone, by setting up a PCR Master Mix as follows:

Reagent	Reagent Volume (µl per sample)
Deionized H ₂ O	7
Adeno-X Screening Primer Mix 3 (10 µM)	0.5
Template (bacterial culture)	5
Terra PCR Direct Red Dye Premix	12.5
Total volume per rxn	25

4. Begin thermal cycling using the following parameters:

1 cycle	98°C for 2 min
30 cycles	98°C for 10 sec
	68°C for 3 min
1 cycle	68°C for 5 min
5. Analyze 5 µl of each PCR reaction on a 1.2% agarose gel.
 - Expected band sizes for clones containing the Adeno-X Control Fragment and for the parental vector alone (non-recombinant) are shown for all of the available pAdenoX vectors in Table 2.
 - The expected band size for successful cloning of your gene of interest will be the size of your gene sequence added to the expected size of the non-recombinant band shown in Table 2 [e.g., for a 3 kb fragment cloned into pAdenoX-ZsGreen1, the expected size of the recombinant will be 4.9 kb (3 kb + 1.9 kb)].
 - Figure 3 shows the screening results for clones obtained when the Adeno-X Control Fragment was inserted into the pAdenoX-PRLS-DsRed-Express vector.

Table 2. Expected Results of PCR Colony Screening Analysis

Vector	Expected Band Sizes When Cloning the Adeno-X Control Fragment (3.0 kb)	
	Recombinant	Non-recombinant
pAdenoX-Tet3G	4.7 kb	1.7 kb
pAdenoX-CMV	4.9 kb	1.9 kb
pAdenoX-DsRed-Express	4.9 kb	1.9 kb
pAdenoX-ZsGreen1	4.9 kb	1.9 kb
pAdenoX-PRLS	3.4 kb	0.4 kb
pAdenoX-PRLS-DsRed-Express	3.4 kb	0.4 kb
pAdenoX-PRLS-ZsGreen1	3.4 kb	0.4 kb

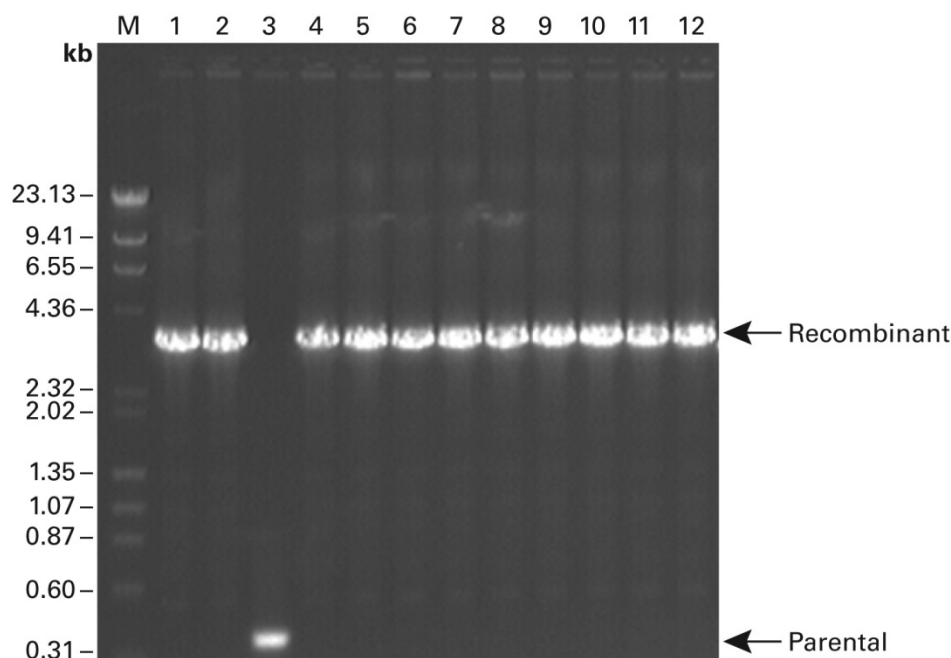


Figure 3. PCR screening of clones using Adeno-X Screening Primer Mix 3. The Adeno-X Control Fragment was cloned into the pAdenoX-PRLS-DsRedExpress vector (as described in Section VII.D), and 12 randomly chosen colonies were subjected to PCR using the Adeno-X PCR Screening Primer Mix 3. Then 5 µl of each sample was analyzed on a 1.2% agarose gel. Expected sizes for positive clones and the parental vector were 3.4 and 0.4 kb respectively. In this example, 92% (11/12) of the clones were positive for the control insert. M=DNA size marker (Phi X174 HaeIII/Lambda HindIII).

C. Protocol: Purifying Recombinant Adenoviral DNA (Midi-Scale)

- After you identify a positive clone by PCR, amplify the clone by inoculating 100 ml of liquid LB/Amp Medium (100 µg/ml ampicillin) with 2–5 ml of fresh, log phase culture—e.g., the culture you set up in Section VIII.B, according to the guidelines specified in Sections V.A & V.B.
- Purify the plasmid using the NucleoBond Xtra Plasmid Midi Kit User Manual (PT4011-1; available at www.takarabio.com/manuals)
 - The expected yield is 30–50 µg plasmid DNA/100 ml of culture.

3. **Restriction Analysis:** When you finish the NucleoBond Xtra Midi purification, **reconfirm** the identity of your recombinant Adeno-X plasmid via **individual digestions with XhoI and NheI**. See Appendix C for estimated sizes.
 - a. **For best resolution:** Digest 1 µg of adenoviral DNA for 3 hrs, and then load half of the digest on a 15 x 5 cm, 0.8–1% agarose gel. Run the gel at ~30 V overnight.
 - b. Typical results are shown in Figure 4. See Appendix A for plasmid maps and restriction sites.

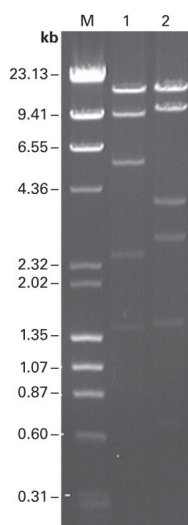


Figure 4. Restriction analysis of pAdenoX DNA. To demonstrate correct restriction digestion and band intensity of the adenoviral DNA, pAdenoXPRLS DsRedExpress was digested with the indicated restriction enzymes according to the protocol and then subsequently analyzed on a 1.2% agarose gel. Lane 1: XhoI. Lane 2: NheI. Lane M: DNA size marker Phi X174HaeIII/Lambda HindIII.

4. **Sequence Analysis:** In addition to reconfirming the identity of your construct by restriction digest, you may use these primers for sequencing of the cloning junctions:
 - **For PT_{RE3G} and P_{CMV} containing vectors (located 3' of cloning site):**
5'-tgtcacaccacagaagtaaggttcc-3'
 - **For Universal vectors (located 5' of cloning site):**
5'-tagtgtggcggaagtgtgatgttgc-3'
 - We also recommend sequencing with insert-specific primers for further confirmation.

IX. Producing Recombinant Adenovirus

A. Protocol: Preparing Recombinant pAdenoX DNA for Transfection

Before pAdenoX DNA can be packaged, the recombinant plasmid must be digested with PacI to expose the inverted terminal repeats (ITRs) located at either end of the adenoviral genome (see Adeno-X Adenoviral Systems 3 Vector Information in Appendix A) and release the adenoviral genome from the plasmid backbone. The ITRs contain the origins of adenoviral DNA replication and must be positioned at the termini of the linear Ad DNA molecule to support the formation of the replication complex (Tamanoi & Stillman, 1982). It is essential to achieve a complete PacI restriction digestion in order to ensure efficient rescue of a recombinant retrovirus.

1. In a sterile 1.5 ml microcentrifuge tube, combine the following reagents:

Reagent	Volume
Sterile deionized H ₂ O	20 µl
Recombinant pAdenoX Plasmid DNA (500 ng/µl)	10 µl
10X PacI Digestion Buffer	4 µl
10X BSA ^a	4 µl
PacI Restriction Enzyme (10 units/ µl)	2 µl
Total Volume per rxn ^b	40 µl

^a Prepare 10X BSA by diluting a small aliquot of 100X BSA with sterile deionized water (1:10).

^b Each 40 µl digest yields enough DNA to transfect one 60 mm culture plate. (The transfection protocol is given in Section IX.B.) To transfect larger cultures, scale the digest proportionally.

2. Mix contents and spin the tube briefly in a microcentrifuge.
3. Incubate at 37°C for 2 hr. Confirm that the PacI digestion is complete by analysis on a 1% agarose gel. The plasmid portion of the recombinant pAdenoX vector will migrate at ~ 3 kb, while the adenoviral genome will not enter the gel, but will remain at the top of the lane.
4. Add 60 µl 1X TE Buffer (pH 8.0) and 100 µl phenol:chloroform: isoamyl alcohol (25:24:1). Vortex gently.
5. Spin the tube in a microcentrifuge at 14,000 rpm for 5 min at 4°C to separate phases.
6. Carefully transfer the top aqueous layer to a clean sterile 1.5 ml microcentrifuge tube. Discard the interface and lower phase.
7. Add 400 µl 95% ethanol, 25 µl 10 M NH₄OAc (or 1/10 volume 3 M NaOAc), and 1 µl glycogen (20 mg/ml). Vortex gently.
8. Spin the tube in a microcentrifuge at 14,000 rpm and 4°C for 5 min.
9. Remove and discard the supernatant.
10. Wash the pellet with 300 µl 70% ethanol.
11. Spin in a microcentrifuge at 14,000 rpm for 2 min.

12. Carefully aspirate off the supernatant.
13. Air dry the pellet for ~15 min at room temperature.
14. Dissolve the DNA precipitate in 10 µl sterile 1X TE Buffer (pH 8.0). Proceed with Section IX.B or store at –20°C.

B. Protocol: Transfecting Adeno-X 293 Cells with Pac I-Digested Adeno-X DNA

1. Plate Adeno-X 293 cells at a density of 1–2 x 10⁶ cells per 60 mm culture plate (approximately 100 cells/mm²) 12–24 hr before transfection.
 - a. For best results, cells should be 50–70% confluent, display a flat morphology, and adhere well to the plate prior to transfection.
 - b. Be sure to include a **transfection control**:
 - i. If you constructed a positive control vector, i.e., pAdenoX-LacZ, which contains the Adeno-X Control Fragment (Section VII.D), seed sufficient plates to produce this virus as well. It is designed as a cloning control but will express LacZ in the Tet-On 3G and CMV Systems—so LacZ expression can be assayed in these control vectors using any standard X-Gal staining protocol (e.g., see Ausubel *et al.*, 1995).
 - ii. The transfection efficiency of any of the ZsGreen1 or DsRed-Express-containing pAdenoX vectors can be monitored via fluorescence microscopy.
2. Incubate the plate(s) at 37°C in a humidified atmosphere maintained at 5% CO₂.
3. Transfect each 60 mm culture plate with 10 µl of PacI-digested Adeno-X DNA. Takara Bio recommends the calcium phosphate transfection method for transfecting large plasmids into Adeno-X 293 cells, using the **CalPhos Mammalian Transfection Kit** (Cat. No. 631312), with the following transfection mix:

Reagent	Volume
PacI-digested pAdeno-X DNA (0.5 µg/µl)	10 µl
Sterile H ₂ O	209 µl
Calcium solution	31 µl
2X HBS	250 µl
Total	500 µl

See the CalPhos Mammalian Transfection Kit User Manual (PT3025-1, available at www.takarabio.com/manuals) for a more detailed protocol.

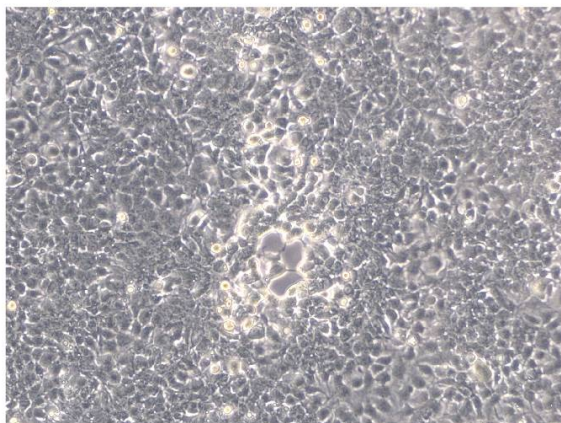
4. One day later, and periodically thereafter, check for the cytopathic effect (CPE) (Figure 5).

Alternatively, Adeno-X GoStix can help rapidly (2–20 min) determine the presence of virus in media post-transfection, using only 20–50 µl of sample (Figure 6). Signal can often be visualized before the outward appearance of CPE.

NOTES:

- Infected cells typically remain intact but round up and may detach from the plate. These changes are collectively referred to as the CPE (see Figure 5).
- The time it takes for CPE to appear depends on the transfection efficiency—it may take up to two weeks for CPE to become evident. Because adenovirus remains associated with cells until late in the infection cycle, high-titer virus is obtained by manually lysing cells with a series of freeze-thaw cycles as explained below in Steps 5–9. It is best to harvest cultures demonstrating a late CPE phenotype (see Figure 5).
- Do not change the medium until CPE is observed and the cells are harvested.

Early CPE



Late CPE

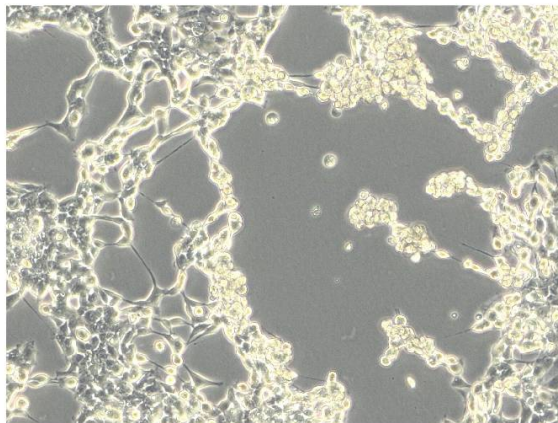


Figure 5. Observing the cytopathic effect when culturing adenovirus.

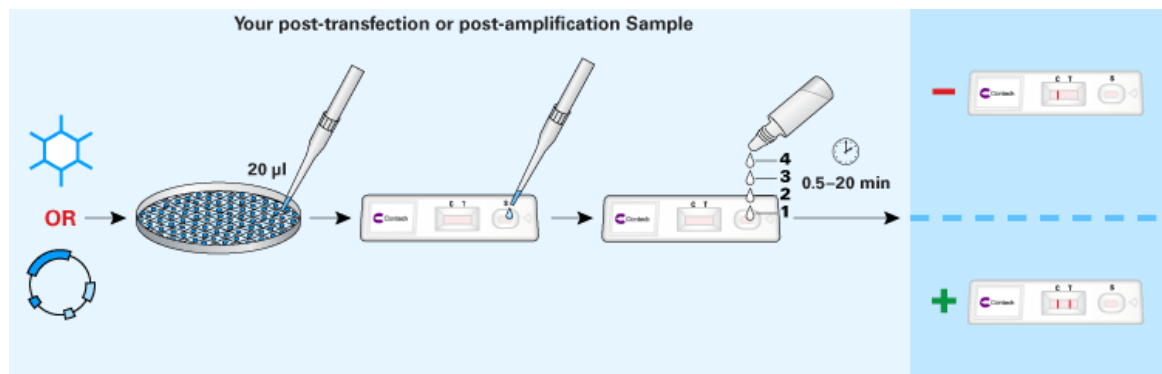


Figure 6. The Adeno-X GoStix protocol takes only 2–20 minutes.

5. One week later, transfer the cells and medium to a sterile, 15 ml conical centrifuge tube. **Do not use trypsin:** Infected cells that remain attached to the bottom or sides of the culture plate can be dislodged into the medium by gentle agitation.
6. Centrifuge the suspension at 1,500 x g for 5 min at room temperature.
7. Resuspend the pellet in 500 µl sterile PBS.
8. Lyse the cells with three consecutive freeze-thaw cycles: Freeze the cells in a dry ice/ethanol bath; thaw the cells by placing the tube in a 37°C water bath. **Do not allow the suspension to reach 37°C.** Vortex the cells each time after thawing.

9. After the third freeze-thaw cycle, briefly centrifuge to pellet debris. Transfer the lysate to a clean, sterile centrifuge tube and either store at -20°C or use immediately in Step 10.
10. Infect a fresh 60 mm culture by adding 250 μl (50%) of the cell lysate from Step 9. Add the lysate directly to the medium and incubate as normal. CPE should be evident within one week.

NOTE: If no CPE appears after one week, the viral titer of the cell lysate from Step 9 may have been too low. Amplify the titer by repeating Steps 5–10.

11. When $>50\%$ of the cells have detached from the plate, prepare a viral stock by following Steps 5–9. Name this stock “Primary Amplification”, and store at -20°C .
 - The Primary Amplification stock is suitable for infecting target cells as described in Section X. We suggest you evaluate the function of this viral stock before preparing High-Titer Stock (Section IX.C).
 - The presence of your recombinant construct can be verified by PCR or Western blotting (Section IX.D).
12. Determine the adenoviral titer using the **Adeno-X Rapid Titer Kit** (Cat. No. 632250), which enables you to determine adenoviral titer using an anti-hexon antibody cell staining assay. Download a free copy of the User Manual (PT3651-1) at www.takarabio.com/manuals to learn more about this product.

Alternatively, you can use Adeno-X GoStix (Cat. No. 632270) to test the quality of the lysate in just 2–20 min. The GoStix can indicate the level of amplification achieved and help to determine if further amplification is required.

C. Protocol: Amplifying Recombinant Adenovirus: Preparing High-Titer Stocks

Prepare high-titer stocks of recombinant adenovirus according to the following protocol:

NOTE: The probability of producing replication competent adenovirus (RCA), although low, increases with each successive amplification. RCA is produced when Adeno-X DNA recombines with E1-containing genomic DNA in Adeno-X 293 cells. For this reason, we suggest you save aliquots of early amplifications. Use early amplification stocks whenever you need to produce additional quantities of adenovirus.

1. About 24 hours before infection, plate Adeno-X 293 cells in a T75 flask. The cell monolayer should be 50–70% confluent when you infect.
2. Incubate the cells overnight at 37°C in a humidified atmosphere maintained at 5% CO_2 .
3. On the following day, replace the medium with 5 ml of fresh growth medium that contains adenovirus: For best results, infect the cells at a multiplicity of infection (M.O.I.) ≥ 5 (i.e., at ≥ 5 ifu/cell). For example, if the T75 flask contains $\sim 5 \times 10^6$ cells, add 2.5×10^7 ifu adenovirus.
4. Incubate for 90 min at 37°C in a humidified atmosphere maintained at 5% CO_2 .
5. Remove the flask and add 10 ml of fresh growth medium.
6. Incubate for 3–4 days at 37°C in a humidified atmosphere at 5% CO_2 .

7. Check for a cytopathic effect. When 50% of the cells have detached, transfer the suspension to a sterile 15 ml conical centrifuge tube. **Do not use trypsin:** Infected cells that remain attached to the bottom or sides of the flask can be dislodged into the medium by gentle agitation.
8. Isolate virus using the freeze-thaw method described in Section IX.B, Steps 5–9. (At Step 7 of Section IX.B, resuspend the pellet in 0.5–1 ml of PBS.)
9. Determine the adenoviral titer using the Adeno-X Rapid Titer Kit (see Section IX.B, Step 12). Expected titer: 10^8 – 10^9 ifu/ml. For quicker, qualitative titer determination, use Adeno-X GoStix.
10. To produce a greater quantity of high-titer adenovirus, use the cell lysate from the first amplification to infect larger cultures (e.g., a series of T175 flasks).
11. Depending on how you intend to use your recombinant adenovirus, you may wish to refine your High-Titer Stock by purification.
 - Use the **Adeno-X Virus Purification Kit** (Cat. Nos. 631532, 631533 & 631032). With this kit, adenovirus can be purified in less than 2 hours, and no ultracentrifugation steps are necessary. The purity is comparable to that achieved with CsCl centrifugation. Download the User Manual (PT3680-1) at www.takarabio.com/manuals to learn more about this product.
 - Alternatively, purify the viral particles on a CsCl gradient. Please consult the following references for protocols on how to purify adenovirus using CsCl gradient centrifugation (Hitt *et al.*, 1998; Hitt *et al.*, 1995; Graham & Prevec, 1991; Spector & Samaniego, 1995; and Becker *et al.*, 1994).

D. Protocol: Evaluating Recombinant Virus: Confirmation of Construct

A number of different methods can be used to verify that the encapsidated adenoviral genome contains a functional copy of your gene:

- The preferred methods detect synthesis of the target protein: e.g., Western blotting, ELISA, or a biochemical assay that specifically measures the enzymatic activity of the expressed protein. If an antibody is not available, Southern blotting can be used to confirm the presence of your gene.
- Alternatively, PCR—e.g., using the Adeno-X Screening Primer Mix 3 or your own gene-specific primers—is a quick and efficient way to evaluate your construct. A small aliquot (e.g., 1 µl) of viral stock can be sampled and used directly for PCR. The high temperatures associated with PCR denature the viral coat proteins and expose the DNA for hybridization with the primers.

X. Infecting Target Cells with Adenovirus & Analyzing Gene Expression

A. Protocol: Infecting Target Cells

Follow these guidelines to optimize the M.O.I. when infecting your target cells:

NOTE: MOI (multiplicity of infection) is defined as the number of infectious units (IFU) per cell at the time of infection, based on the adenovirus titer (IFU/ml) as determined by the **Adeno-X Rapid Titer Kit** (Cat. No. 632250).

- We recommend infecting target cells at an M.O.I. of 10–100 ifu/cell.
- The M.O.I. needed to efficiently transmit your gene of interest to a particular host cell population depends on the biological properties of the target cell line and, therefore, must be determined empirically.
- An excessively high M.O.I. can be toxic to cells; however, an extremely low M.O.I. may not enable you to accurately evaluate the phenotype of an infected cell line.
- To infect some lymphoid cell lines, you may need to use higher M.O.I.s—e.g., 1,000 ifu/cell. To infect the maximum number of cells, use the smallest volume needed to cover the cells.

Infect your target cells as follows:

1. Plate target cells on 6-well plates 12–24 hr before infection. The seeding density will depend on the growth characteristics of your cell line.

NOTE: Always use filtered pipette tips when handling viruses and cells.

Positive control infection: If you constructed a *lacZ* adenoviral control, be sure to seed enough plates to allow for this infection.

2. The next day, remove the growth medium and add 1.0 ml of virus (diluted to achieve the desired M.O.I.) to the center of each plate. Tip the plates to spread the virus evenly.
3. Cover the plates and incubate the cells in a humidified CO₂ (5%) incubator at 37°C for 4 hours to allow the virus to infect the cells.
4. Add fresh complete growth medium. Incubate in a humidified CO₂ incubator at the temperature appropriate for your cell line.
5. Analyze gene expression at different time points following viral infection. In general, detectable levels of your gene product should be evident 24–48 hr after infection.

B. Analyzing Beta-Galactosidase Expression in Infected Cells

The expression of beta-galactosidase in adherent cells infected with Adeno-X-LacZ can be observed by staining with X-Gal using our **Beta-Galactosidase Staining Kit** (Cat. No. 631780). To quantify beta-galactosidase expression, we recommend using our **Luminescent Beta-Galactosidase Reporter System 3** (Cat. No. 631713).

C. Protocol: Inducible Expression using Tet-On® 3G

The following is a typical procedure for use with the Tet-On 3G Inducible Adeno-X Adenoviral System 3 (Cat. No. 631180).

General Considerations:

- We recommend infecting target cells at an M.O.I. of 10–100 ifu/cell. It is important to test multiple M.O.I.s to ensure that the highest fold induction is achieved.
- The M.O.I. needed to efficiently transmit your gene of interest to a particular host cell population depends on the biological properties of the target cell line and, therefore, must be determined empirically.
- An excessively high M.O.I. can be toxic to cells; however, an extremely low M.O.I. may not enable you to accurately evaluate the phenotype of an infected cell line.
- To infect some lymphoid cell lines, you may need to use higher M.O.I.s—e.g., 1,000 ifu/cell. To infect the maximum number of cells, use the smallest volume needed to cover the cells.

Procedure:

- Plate target cells on 6-well plates 12–24 hr before infection. The seeding density will depend on the growth characteristics of your cell line.

NOTE: Always use filtered pipette tips when handling viruses and cells.

Positive control infection: If you constructed a *lacZ* adenoviral control, be sure to seed enough plates to allow for this infection.

- The next day, remove the growth medium and add 1.0 ml of virus (diluted to achieve the desired M.O.I.) to the center of each plate. Tip the plates to spread the virus evenly.
- Cover the plates and incubate the cells in a humidified CO₂ (5%) incubator at 37°C for 4 hours to allow the virus to infect the cells.
- Add fresh complete growth medium with or without 10–1,000 ng/ml doxycycline. Incubate in a humidified CO₂ incubator at 37°C.

NOTES:

- Doxycycline must be added to the medium at least every 48 hr to regulate gene expression.
- For most experiments, 100 ng/ml of Dox will induce high levels of expression. You can determine empirically which is the minimal amount required for maximal induced expression.
- As a general rule, very high M.O.I.s will yield higher maximal expression in the presence of Dox (Figure 7), but also higher background expression in the absence of Dox. For the tightest control, we recommend that you test a range of M.O.I.'s for each new cell line that you use.

- Analyze gene expression at different time points following viral infection. In general, Dox-induced expression peaks 24–48 hr after infection.

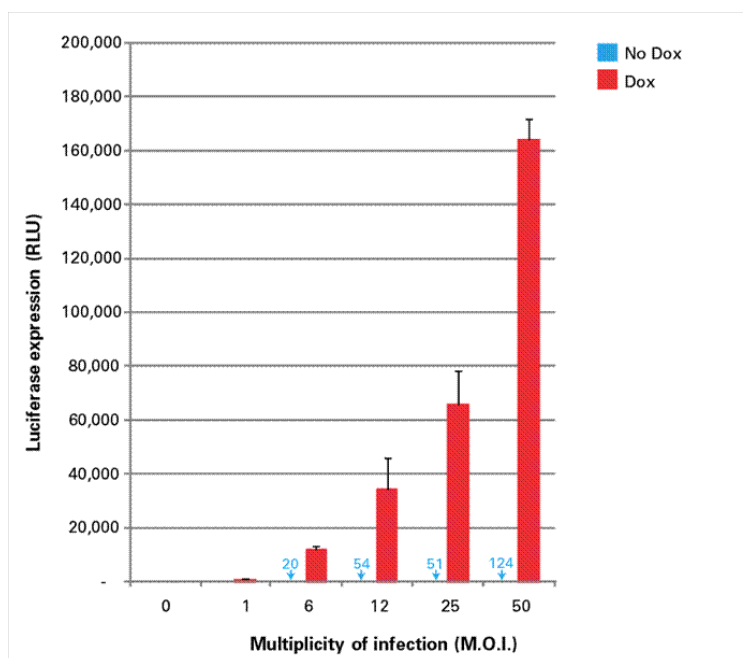


Figure 7. Induced luciferase expression in HeLa cells infected with increasing amounts of Adeno-X Tet-On 3G Luciferase virus. HeLa cells were infected with varying M.O.I.s of pAdenoX Tet-On 3G adenovirus that expresses luciferase. After 4 hours, the media was replaced with fresh media +/- doxycycline (1µg/ml). Cells were harvested 72 hr later and assayed for luciferase activity. Maximal expression increases with increasing M.O.I., which also results in a slight increase in background expression.

XI. References

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XII. Troubleshooting

A. In-Fusion Cloning

1. No or Few Colonies Obtained from Transformation		
Description of Problem	Possible Explanation	Solution
Low transformation efficiency	Transformed with too much In-Fusion reaction	Do not add more than 5 µl of the In-Fusion reaction to 50 µl of competent cells.
	Competent cells are sensitive to the In-Fusion enzyme	We recommend using Stellar chemically competent cells with the pAdenoX vectors.
	Bacteria were not competent	Check transformation efficiency. You should obtain $>1 \times 10^8$ cfu/µg; otherwise use fresh competent cells. Use an alternate strain of <i>E.coli</i> . We recommend Stellar chemically competent cells.
	Wrong antibiotic	Plate on LB agar containing 100 µg/ml of ampicillin.
Low-quality DNA fragments	Low DNA concentration in reaction	The amount of PCR fragment used was too low.
	Gel purification introduced contaminants	The total volume of purified vector and insert should not exceed 5 µl. When possible, optimize your PCR amplification reactions such that you generate pure PCR products.
	Primer sequences are incorrect	Check primer sequences to ensure that they provide 15 bases of homology with the region flanking the insertion site as in Section VII.B.

B. In-Fusion Cloning...continued

2. Large Numbers of Colonies Contained No Insert		
Description of Problem	Possible Explanation	Solution
Large numbers of background colonies containing no cloned insert	Contamination of the In-Fusion reaction by a plasmid with the same antibiotic resistance as the pAdenoX cloning vector	If your insert was amplified from a plasmid, closed circular DNA may have been carried through purification and contaminated the cloning reaction: a) To ensure the removal of any plasmid contamination, we recommend linearizing the template DNA before performing PCR. b) If you spin column-purify your insert, treating the PCR product with DpnI before purification will help to remove contaminating template DNA.
	Plates were too old or contained the incorrect antibiotic	Make sure that your antibiotic plates are fresh (<1 month old). Confirm that the antibiotic is compatible with the pAdenoX vector.

3. Clones Contained the Incorrect Insert		
Description of Problem	Possible Explanation	Solution
Large number of colonies contained the incorrect insert	Your PCR product contained nonspecific sequences	If your PCR product is not a single distinct band, then it may be necessary to gel-purify the PCR product to ensure cloning of the correct insert.
Restriction analysis of DNA prepared from large-scale culture reveals more bands than expected	DNA contamination	Retransform a fresh aliquot of competent <i>E. coli</i> . Inoculate a 5 ml culture; incubate 6–8 hr; then immediately transfer 2–3 ml to 100 ml of fresh LB/Amp. Incubate overnight and purify as in Section VIII.C.
Restriction enzymes do not cut DNA prepared from large-scale liquid culture	Inhibition of enzyme activity by contaminants derived from the bacterial culture	Remove all traces of culture medium from the bacterial pellet before beginning the NucleoBond purification protocol.

C. Producing Recombinant Adenovirus

Description of Problem	Possible Explanation	Solution
No viral particles were produced	Poor transfection efficiency	Check the transfection efficiency, using a suitable control plasmid. We normally observe transfection efficiencies in the range of 20–30% when we transfect a 60 mm plate with 5 µg of pAdenoX plasmid DNA.
	The Adeno-X 293 cell culture used for transfection may be too dense	- Adjust seeding density of cells to optimize confluency at time of transfection. - Check for abnormal growth characteristics and morphology. - Start a fresh culture of low passage cells (e.g., $p \leq 50$). For best results, the Adeno-X 293 cells used in transfections should be at low passage, and approximately 50–70% confluent at the time of transfection.
	Too little or too much pAdenoX DNA used	Titrate the amount of <i>Pac</i> I-digested Adeno-X recombinant DNA needed to achieve maximal transfection efficiency; as a starting point we recommend using 2–5 µg of Adeno-X linearized DNA for a 60 mm plate of Adeno-X 293 cells (50–70% confluent).
High rate of cell death	The protein encoded by your gene insert may be toxic to Adeno-X 293 cells.	Try using the Adeno-X Tet-On 3G Expression System. With this system you are able to modulate the expression of your gene.

D. Infecting Target Cells with Adenovirus

Description of Problem	Possible Explanation	Solution
High rate of cell death	The multiplicity of infection (M.O.I.) may be too high.	Infect at a lower M.O.I.
	Your gene insert may be toxic to host cells	Try using the Adeno-X Tet-On 3G Expression System. With this system you are able to modulate the expression of your gene
	The crude cell lysate was used for transduction	Purify your adenoviral stock using our Adeno-X Purification products
Low expression of gene insert	Low infection frequency of target cell population	- Infect at higher M.O.I. - Retiter adenovirus stock. - Adjust seeding density of cells to optimize confluency at time of infection. - Check for abnormal growth characteristics and morphology. Establish fresh cultures if abnormalities are observed.
	Target cells are not susceptible to infection by adenovirus	Try using lentiviral or retroviral-mediated gene delivery and expression.

E. Appendix A: Adeno-X Adenoviral Systems 3 Vector Information

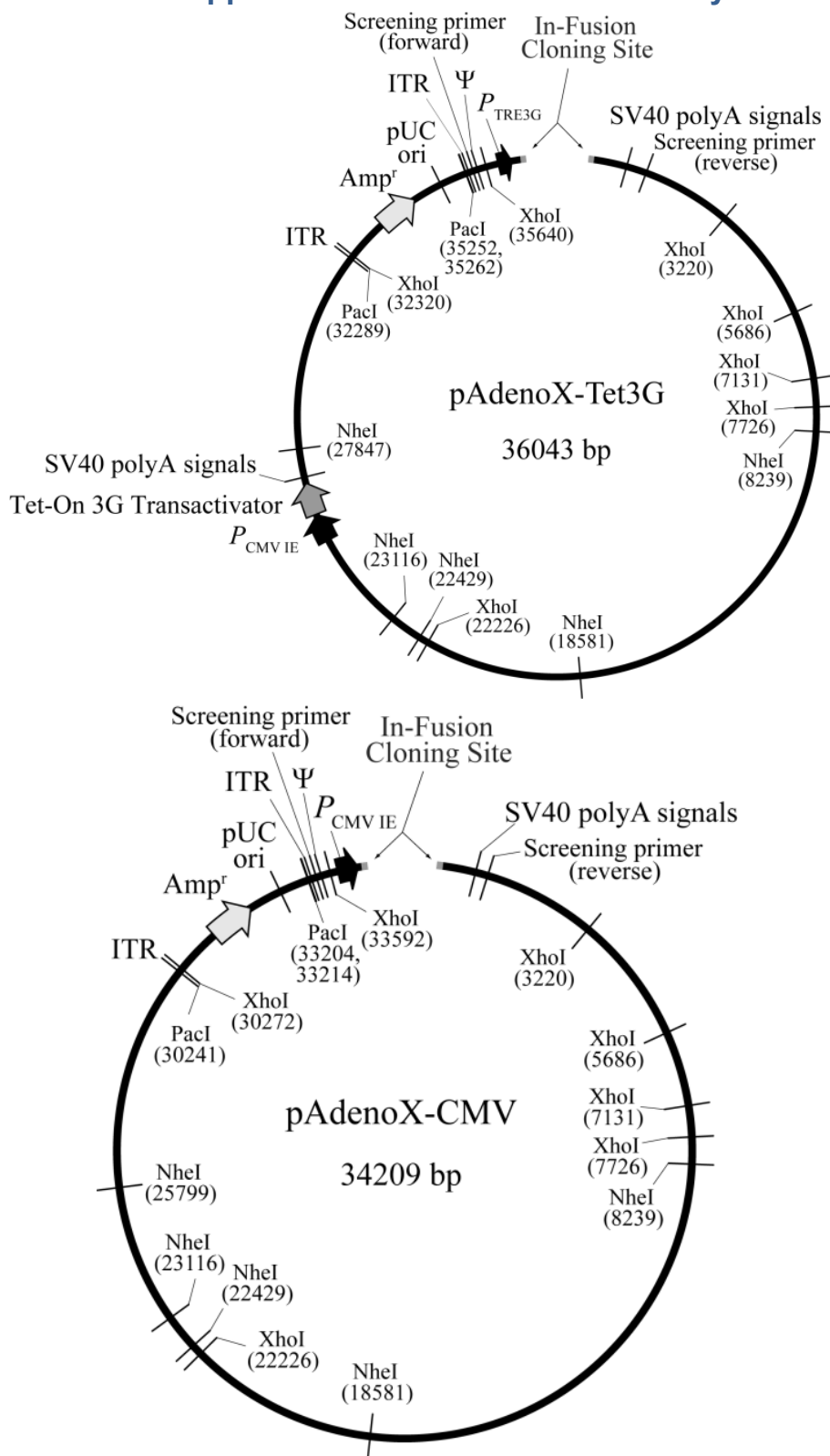


Figure 8. pAdenoX-Tet3G (Linear) Vector and pAdenoX-CMV (Linear) Vector maps.

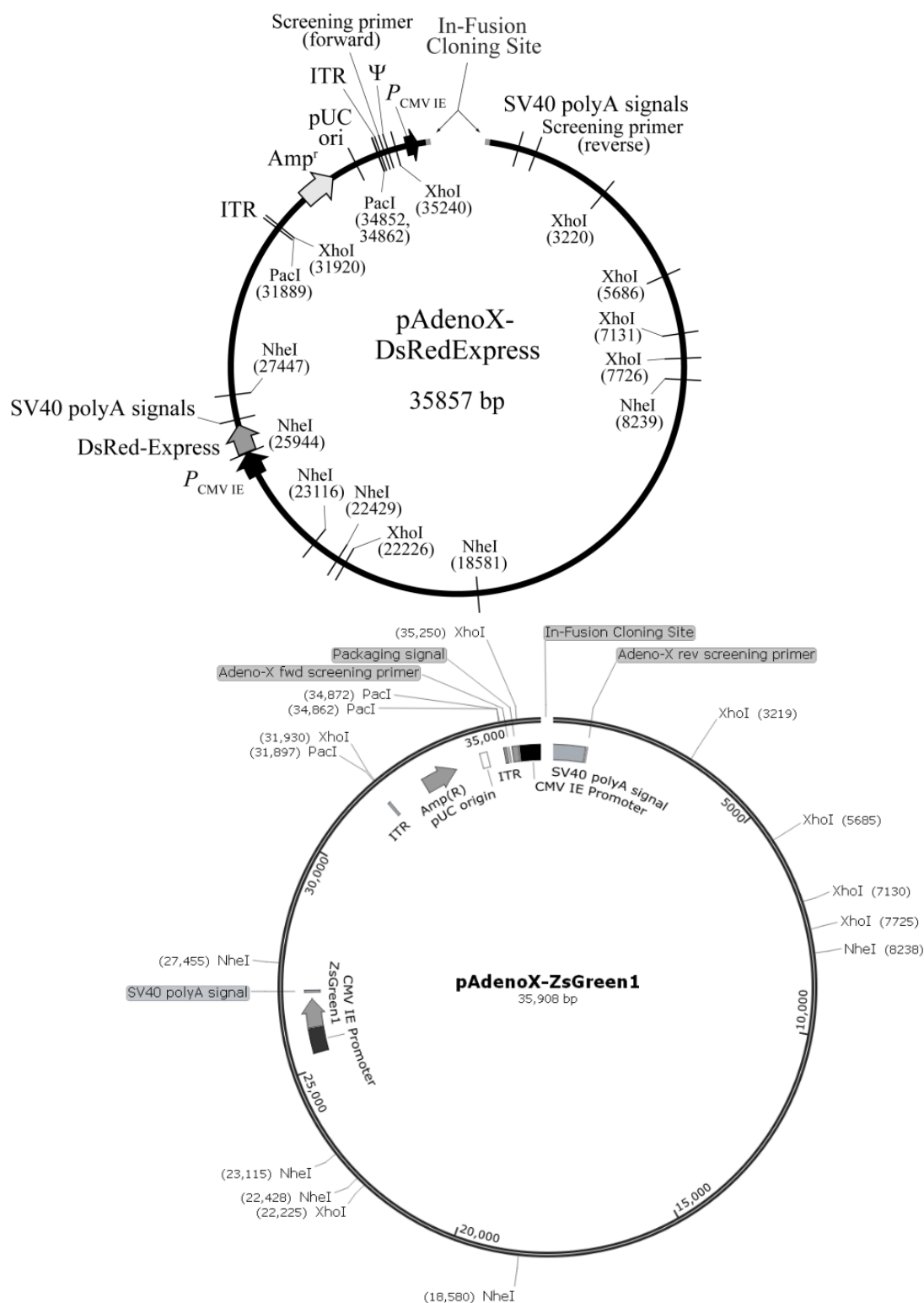


Figure 9. pAdenoX-DsRedExpress (Linear) Vector and pAdenoX-ZsGreen1 (Linear) Vector maps.

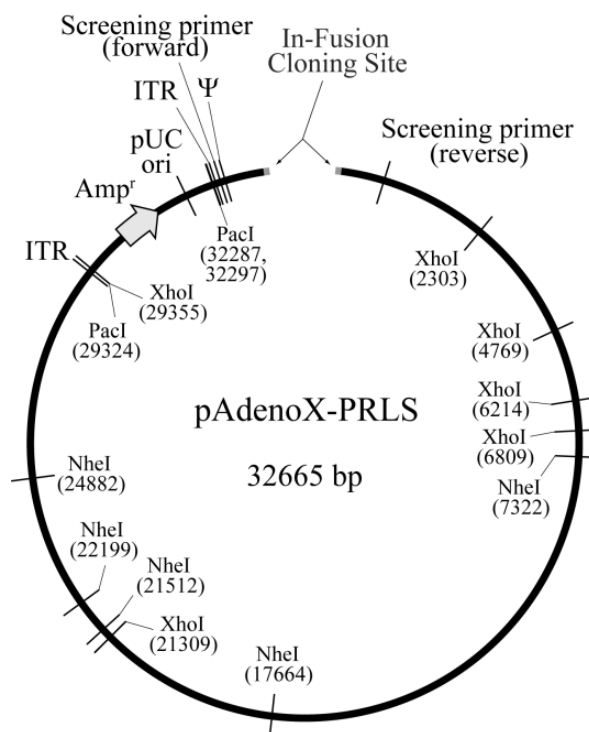


Figure 10. pAdenoX-PRLS (Linear) Vector map.

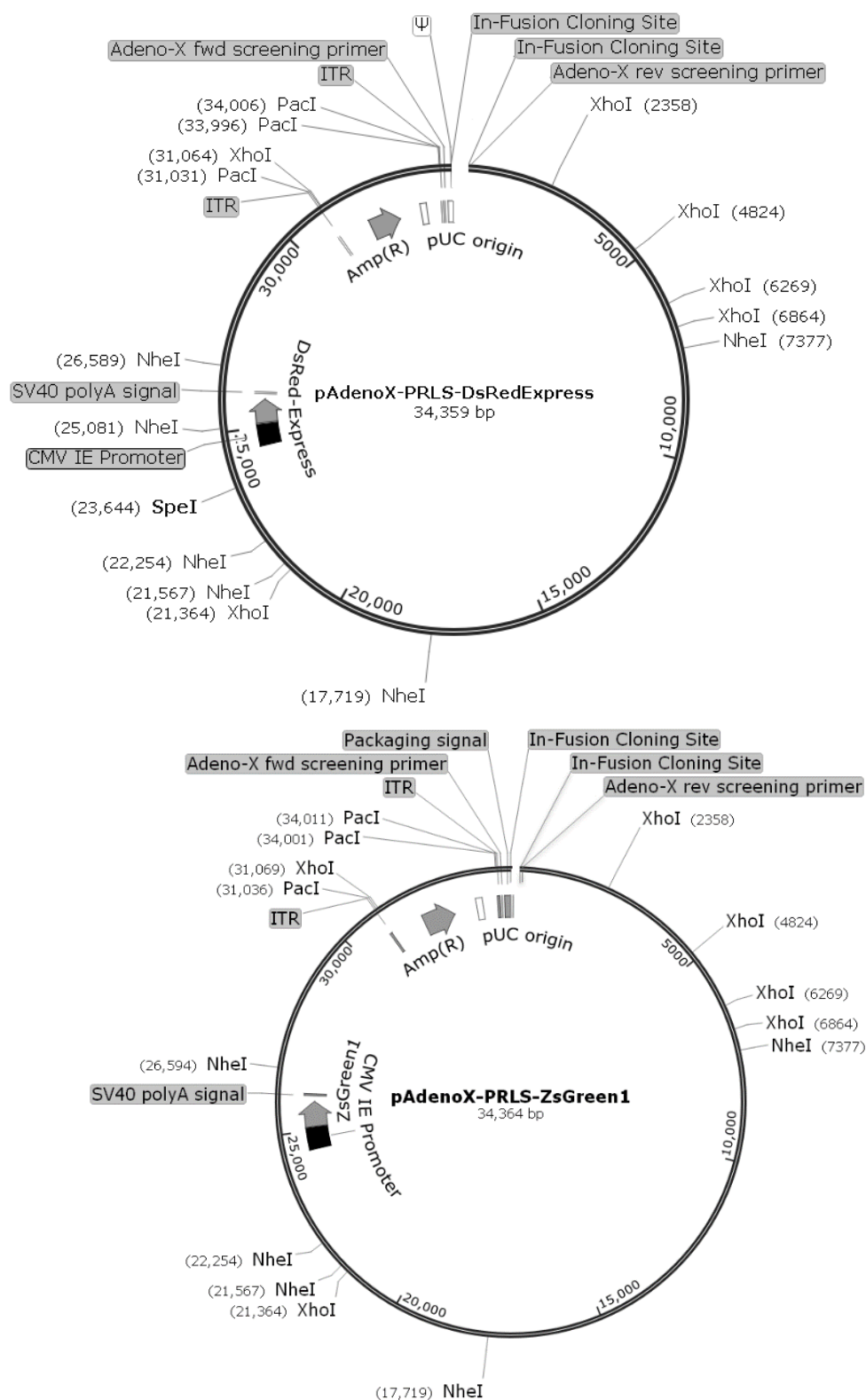


Figure 11. pAdenoX-PRLS-DsRedExpress (Linear) Vector and pAdenoX-PRLS-ZsGreen1 (Linear) Vector maps.

F. Appendix B: Multiple Fragment Cloning into pAdenoX Vectors

The full capabilities of adenoviral delivery can now be realized through the ability of In-Fusion to efficiently and seamlessly assemble all the necessary expression elements into an adenoviral vector. The protocol is simply an extension of the single fragment protocol (Figure 2); all that is required is that the primers for the individual PCR products share 15 bases of homology with the adjacent fragment. (see Figure 12).

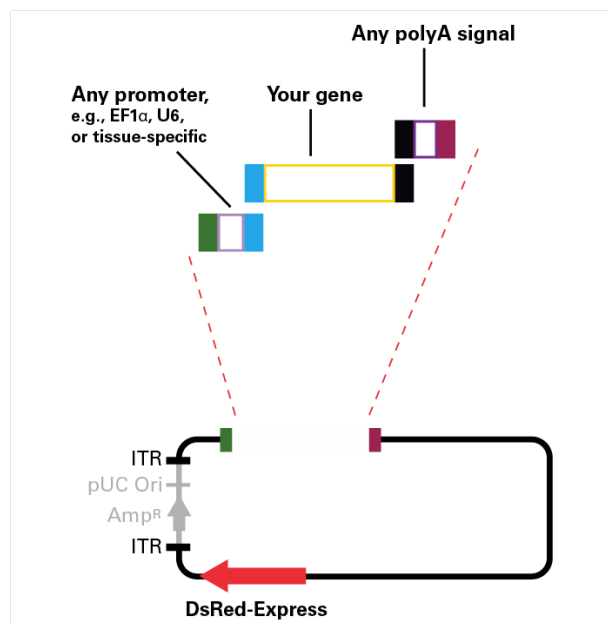


Figure 12. Multiple fragment cloning in the Universal Red System.

Perform the In-Fusion reaction for multiple fragment cloning as follows:

NOTES:

- Whether you are cloning in 2 or 3 fragments, use 50 ng of each PCR insert in a total rxn volume of 10 μ l.
- Due to the complex nature of the cloning, colony number will decrease compared to cloning of single inserts.

1. Prepare 0.2 ml PCR tubes to set up the In-Fusion reaction:

Reagent	Reagent Volume (μ l per sample)	
	Cloning Reaction	Control Reaction
PCR inserts (50 ng each) + deionized H ₂ O	7	0
Additional deionized H ₂ O	0	5
Linearized pAdenoX vector (200 ng/ μ l)	1	1
Adeno-X Control Fragment (50 ng/ μ l)	0	2
5X In-Fusion Snap Assembly Master Mix	2	2
Total volume per rxn	10	10

2. Incubate the reaction for 15 min at 50°C, then place on ice.
3. Proceed with the Transformation Procedure in Section VIII.A. You can store the cloning reactions at –20°C until you are ready.

G. Appendix C: Expected Fragment Sizes for NheI or XhoI digest of pAdenoX Vectors

Table 3. Expected Fragment Sizes (bp) from an NheI or XhoI digest of pAdenoX Vectors. The fragment sizes are based on circular vectors lacking an insert. The shaded values will differ based on the size of the insert that is cloned.

Vector	Linear	NheI	XhoI
pAdenoX-Tet3G	36043	687	595
		3848	1445
		4731	2466
		16435	3623
		10342	3320
			10094
			14500
pAdenoX-CMV	34209	687	595
		3848	1445
		4340	2466
		10342	3320
		16691	3877
			7505
			14500
pAdenoX-ZsGreen1	35908	687	595
		3848	1445
		4340	2466
		10342	3320
		16691	3837
			9703
			14500

pAdenoX-DsRedExpress	35857	687	595
		1503	1445
		2828	2466
		3848	3320
		10342	3837
		16649	9694
			14500
pAdenoX-PRLS	32665	687	595
		2683	1445
		3848	2466
		10342	5613
		15105	8046
			14500
pAdenoX-PRLS-ZsGreen1	34364	687	595
		3848	1445
		4340	2466
		10342	5653
		15147	9705
			14500
pAdenoX-PRLS-DsRedExpress	34359	687	595
		1508	1445
		2827	2466
		3848	5653
		10342	9700
		15147	14500

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This document has been reviewed and approved by the Quality Department.