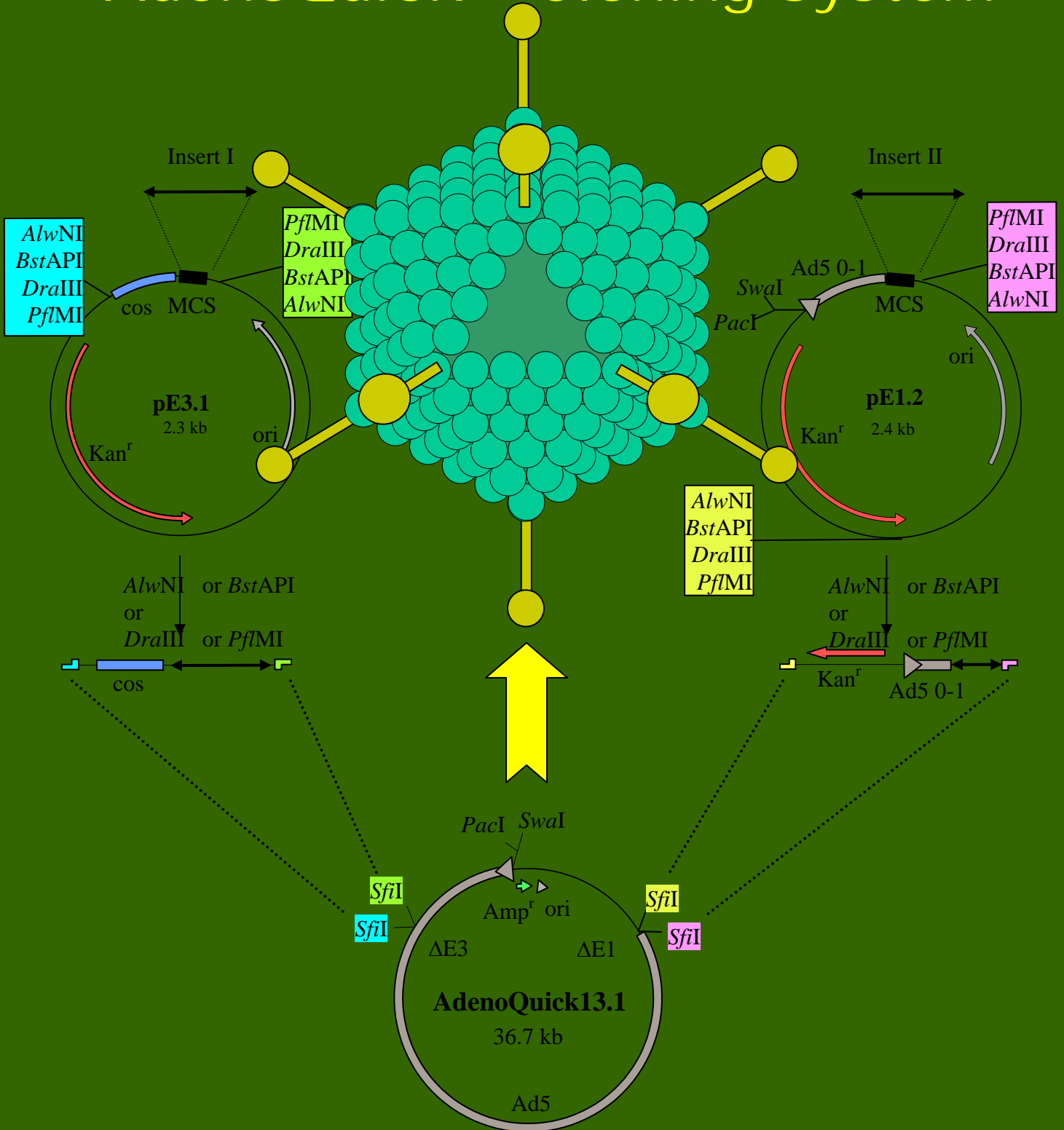


# CrAd Construction using the AdenoQuick™ Cloning System



## Important Notice

Before opening the kit:

- Read our License Agreement (p. 3)
- Read the entire manual, especially the chapter “Safety Considerations about Adenoviruses” (p. 11).
- Become familiar with the different techniques used, especially those related to the manipulation of adenovirus.
- Inquire about regulatory rules for recombinant DNA with your institution or company. If none exists, refer to the NIH guidelines for research involving recombinant DNA molecules (<http://www4.od.nih.gov/oba/rdna.htm>)

## License Agreement

The purchased product includes one or more of the Materials identified in Section G (p. 35 of this manual). In the course of carrying out experiments, it is anticipated that the Recipient will create various Derivatives of the Materials. Derivatives are defined as other materials including, without limitation, DNA, plasmids and viral vectors.

By opening the kit and using these products, the Recipient agrees with the following:

1. The Recipient agrees to use the Materials and Derivatives solely for experimental purposes. These products will not be used, under any circumstances, in humans or for any human diagnostic or commercial purposes.
2. The Recipient agrees that the Materials and Derivatives will be used only at the Recipient's facilities and only by the Recipient or under the Recipient's direct supervision. The Recipient agrees to refrain from distributing or releasing samples or copies of the Materials or Derivatives to any third party.
3. The Recipient shall hold O.D.260 Inc. harmless for any damages which may be alleged to result from the transfer, storage, handling, use or disposal of the Materials or Derivatives thereof, subject to any relevant state or federal governmental laws or regulations.
4. In view of the Materials' experimental nature, O.D.260 Inc. provides no warranty, express or implied, including any warranty of merchantability or fitness for a particular purpose or warranty against infringement.
5. All unused supplies of the Materials and Derivatives will, at O.D.260 Inc.'s option, be destroyed or returned to O.D.260 Inc., when the investigation for which they have been purchased discontinues or is terminated.
6. The Recipient agrees to comply with all laws and regulation for the handling and use of the Materials. The Recipient agrees to follow the US National Institute of Health (NIH) guidelines, including the NIH Guidelines for Research involving Recombinant DNA Molecules, or applicable equivalents for safe use of biologicals, including Adenovirus-based biologicals.
7. This agreement is between the Recipient and O.D.260 Inc. but also applies to members of the Recipient's direct research staff.

If these terms are acceptable to you, then please continue. If you do not agree with these terms, please contact O.D.260 Inc. at (208) 345-7369 to arrange for

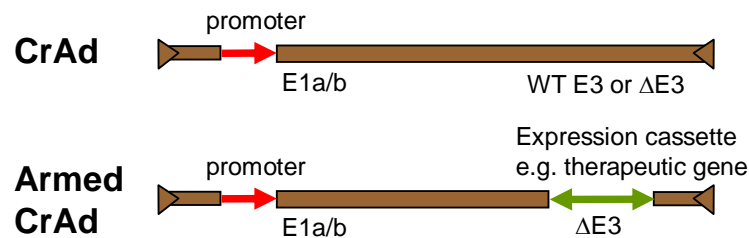
a product credit or refund, and return the unopened kit within 10 days of receipt. Please note that products may not be returned without prior authorization from O.D.260 Inc.

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## A. Introduction

The AdenoQuick cloning system was designed to construct first-generation adenoviral vectors containing one or two expression cassettes in the E1 and E3 regions. The method is based on the construction of a large plasmid/cosmid that contains the entire sequence of the recombinant virus and the transfection of the linearized DNA into helper cells in order to recover the virus. The system was adapted for the construction of conditionally replicative adenoviruses (CrAds), in which a specific promoter directs the transcription of the E1 region. The E3 region can be intact (wild-type), deleted (2.7 kb), or substituted with an expression cassette of interest, such as a therapeutic gene, so that “armed” CrAds can be made (Figure 1).



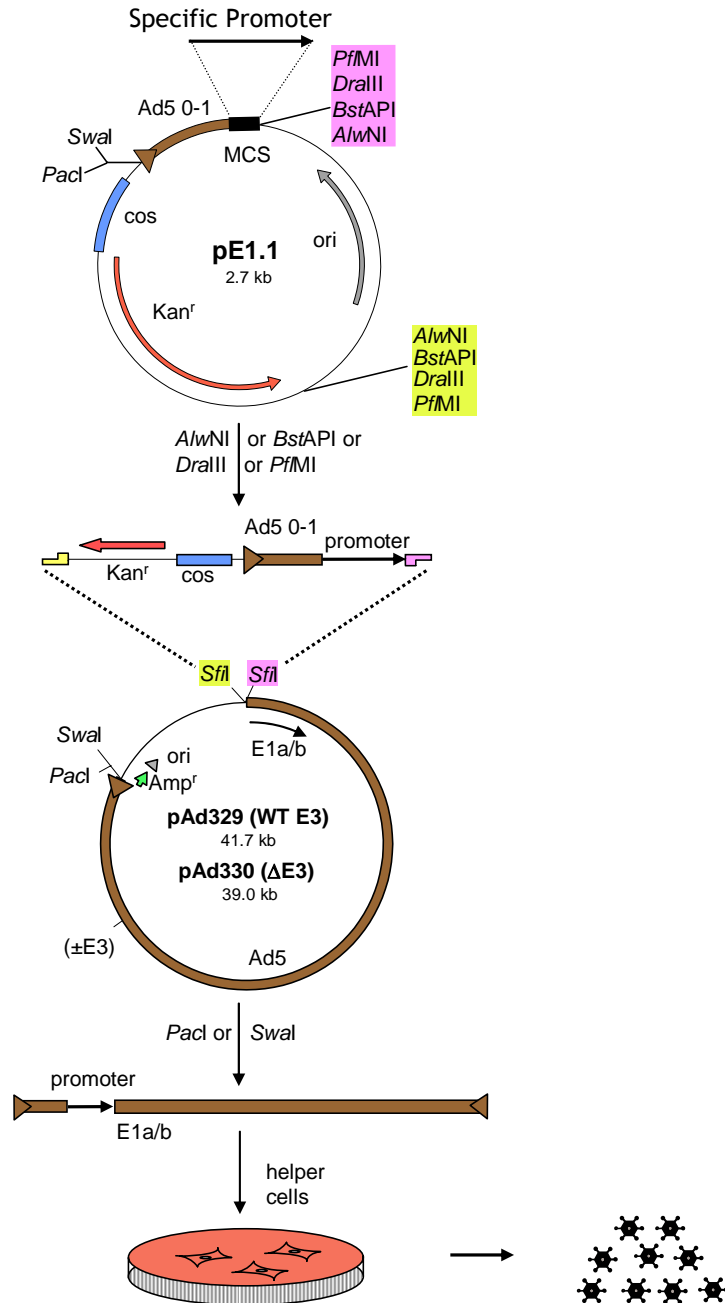
**Figure 1: Typical Structure of Conditionally Replicating Adenoviruses (CrAd) that can be constructed using the AdenoQuick cloning system.** A promoter of interest (e.g. tumor-specific promoter) is inserted in front of the E1 region. The E3 region can be left intact (wild-type), deleted (2.7 kb), or an expression cassette such as a therapeutic gene can be inserted into it in order to construct an “armed” CrAd.

CrAd construction using the AdenoQuick system is detailed in Figure 2 and Figure 3. Three steps are required:

1. The expression cassette or promoter sequences are cloned into shuttle vectors that contain bacterial selectable markers (pE1.1, pE1.2, pE3.1)
2. Using a versatile set of restriction enzymes that allow for directional cloning, the expression cassettes or promoter sequences and the selection markers are transferred into large plasmids containing the adenoviral sequences, including the E1a/b region (pAd328, pAd329, and pAd330).
3. The resulting plasmid/cosmid is linearized and transfected into helper cells to generate the virus.

In addition to being the only system adapted for the construction of bipartite adenoviruses and (armed) CrAds, the AdenoQuick system has the following advantages, which facilitate greatly the cloning procedures:

1. Cosmid technology is particularly well suited for the cloning of the 36 kb-long adenoviral genome. Because phage  $\lambda$  packages DNA's ranging from 39 to 54 kb, the method selects clones containing full-size genomes.
2. No homologous recombination event is necessary, thus:
  - There is no danger of an unpredicted recombination in *E. coli* that would not be detected by restriction analysis and would render the DNA non-infectious.
  - There is no need for the transformation of a *recA*<sup>+</sup> strain (eg: BJ5183) for the recombination and the subsequent transfer to a *recA endA* strain for plasmid preparation. Phage  $\lambda$  infection can be performed directly into *recA endA* strains such as DH5 $\alpha$ , XL-1 blue or Top10.
3. The method allows for the simultaneous insertion of two independent transcription units at different locations in the genome (E1 or E3 regions).
4. The cosmid construction is very efficient:
  - Each expression cassette is linked to a positive selection marker (Kan<sup>r</sup> or *cos* site). Therefore its presence is ensured in the resulting construct.
  - The restriction sites used for cloning (*Sfi*I, *A*luNI...) generate different sticky ends that allow for directional cloning.
  - The cosmid construction via packaging into phage  $\lambda$  produces generally hundreds of clones, with practically 100% efficiency. The cosmid construction via electroporation is about 70% efficient.
  - One milliliter bacterial culture can yield up to 3  $\mu$ g cosmid DNA.
5. The method is fast:
  - Two expression units can be introduced simultaneously into the viral genome.
  - Plaques usually appear 7 to 10 days after transfection, sometimes as early as 4 days.



**Figure 2: Construction of Conditionally-Replicative Adenoviruses using the AdenoQuick Cloning System.** The promoter of interest is cloned into the multiple cloning site (MCS) of pE1.1, downstream from the Ad5 left ITR (brown triangle) and packaging signal (brown bar) (Ad5 map unit 0-1). A DNA fragment containing the kanamycin-resistance gene,  $\lambda$  cos site, the adenovirus sequences and the promoter of interest is excised from the plasmid by restriction digestion with *Alw*NI, *Bst*API, *Dra*III or *Pfi*MI, and ligated with *Sfi*I-digested pAd329 (WT E3) or pAd330 ( $\Delta$ E3). The ligated DNA is transferred into *E. coli* via packaging into phage  $\lambda$  or by electroporation. The resulting cosmid/plasmid is linearized with either *Pac*I or *Swa*I and transfected into helper cells (e.g. HEK 293 cells) in order to recover the virus.

## 6. The method is versatile:

- Four different enzymes are available to excise the expression cassettes from the intermediate vectors.
- Two rare-cutting enzymes are available to linearize the cosmid before transfection into 293 cells. Therefore, this method is likely to be useful in a very large number of applications.
- The reconstitution of the genome of the recombinant virus in a cosmid can be performed either via packaging into phage  $\lambda$  or by electroporation.

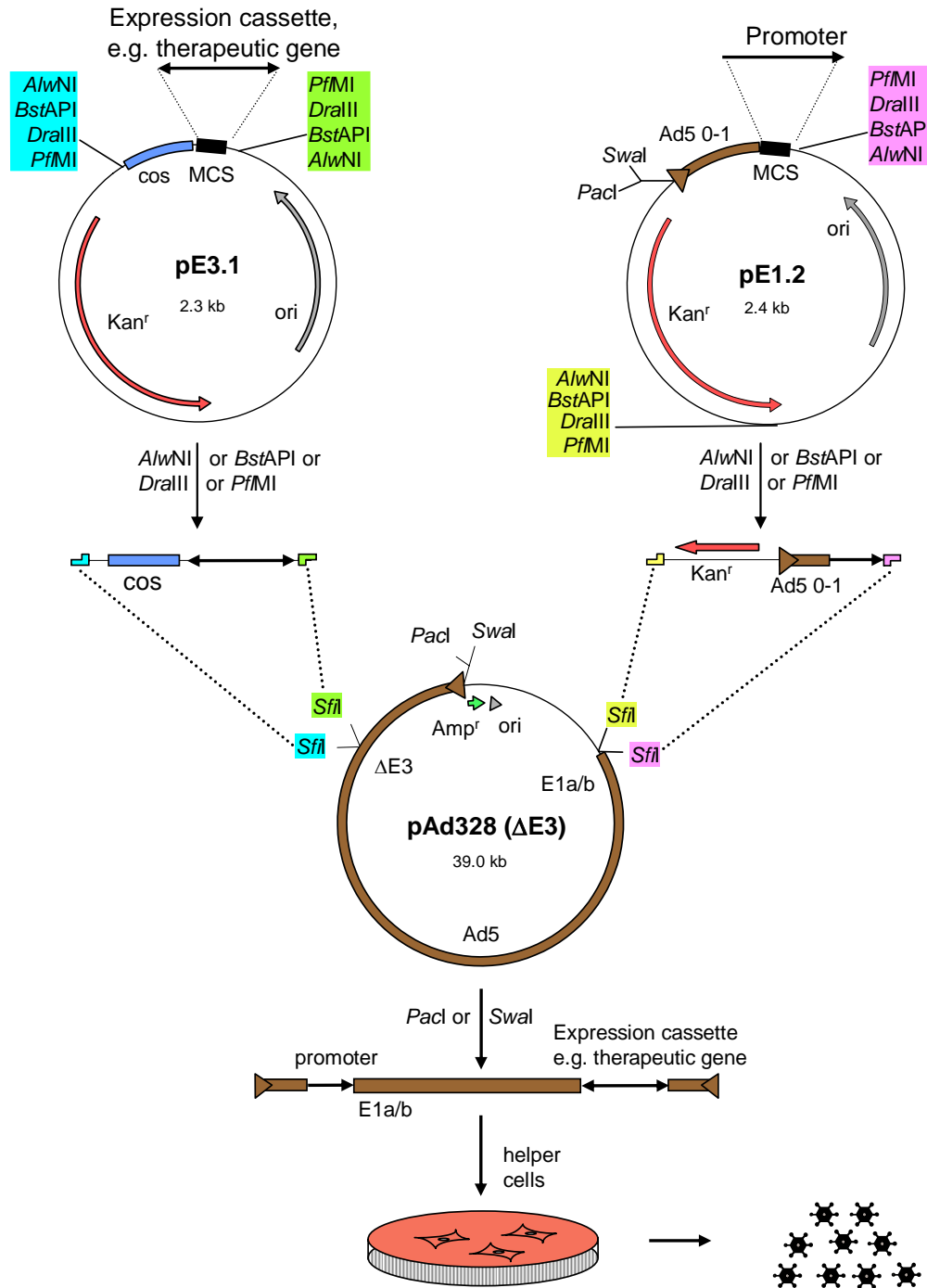
## 7. Compared to other techniques, this method requires less “hand-on” time:

- Compared to the techniques that use viral DNA as donor sequences, this method is not subject to contamination with parental virus and therefore a limited number of plaques will have to be analyzed, unless the virus is unstable.
- Compared to the other methods that reconstitute the genome of the recombinant virus in a plasmid or cosmid, this method is so efficient in generating the cosmid that a medium-scale bacterial culture (midi-prep) can be initiated directly from a single clone without verifying its identity beforehand (mini-prep).

As shown in Figures [1](#) and [2](#), the AdenoQuick cloning system includes three shuttle vectors and three large adenovirus plasmids for constructing CrAds. The table below offers a synopsis of the different cloning possibilities, including the E3 region status, maximum transgene capacity and the different combinations of shuttle vector/adenovirus plasmid.

**Table 1: Overview of the Various Cloning Possibilities Offered by the AdenoQuick System for CrAd construction.**

| E3 region status                 | Replication-competent adenovirus with a specific promoter in the E1 region |                      | Replication-competent adenovirus with a specific promoter in the E1 region and an expression cassette in the E3 region |
|----------------------------------|--|----------------------|--|
|                                  | WT E3  | $\Delta$ E3 (2.7 kb) | $\Delta$ E3 (2.7 kb)   |
| Max. promoter/transgene capacity | 2.1 kb   | 4.8 kb               | 4.6 kb   |
| Adenoviral plasmid               | pAd329   | pAd330               | pAd328   |
| E1 region-shuttle                | pE1.1  | pE1.1                | pE1.2  |
| E3 region-shuttle                | N/A  | N/A                  | pE3.1  |



**Figure 3: Construction of Armed Conditionally-Replicative Adenoviral Vectors using the AdenoQuick Cloning System.** The promoter and expression cassette of interest are cloned into the multiple cloning sites (MCS) of pE1.2 and pE3.1. The resulting plasmids are digested with *A*l*u*NI, *B*stAPI, *D*raIII or *P*f*M*I, and the DNA fragments containing the expression units and the positive selection markers (λ cos site or Kan<sup>r</sup>) are ligated with *S*f*I*-digested pAd328. The ligated DNA is transferred into *E. coli* preferentially via packaging into phage λ. The resulting cosmid is linearized with either *P*acI or *S*wal and transfected into helper cells (e.g. HEK 293 cells) in order to recover the virus.

## **B. Safety Considerations about Adenovirus**

### **1. Epidemiology**

Human adenoviruses belong to the genus *Mastadenovirus*, of which 41 serotypes are currently recognized. Adenovirus infections occur most frequently in infants and children. Infections are less frequent in adults, accounting for less than 2 percent of respiratory illness. Nearly 100% adults have serum antibody against multiple serotypes, indicating that infection is common in childhood. Types 2, 3, and 5 are the most frequent isolates obtained from children. Certain adenovirus serotypes (3, 4, 7, 14, 21) are associated with outbreaks of acute respiratory disease. Some adenovirus types can induce oncogenic transformation, and tumor formation has been observed in rodents, but despite intensive investigation, adenoviruses have not been associated with tumors in humans.

Transmission of adenovirus infection can occur by inhalation of aerosolized virus, by inoculation of virus in conjunctival sacs, and probably occurs by the fecal-oral route as well.

### **2. Clinical Manifestations**

In adults, the most frequently reported illness has been acute respiratory disease caused by adenovirus types 4 and 7. This illness is marked by a prominent sore throat and the gradual onset of fever. Cough is almost always present, and coriza and regional lymphadenopathy are also frequently seen.

Adenoviruses have also been associated with a number of non-respiratory tract diseases, including acute diarrheal illness in young children caused by adenovirus types 40 and 41, and hemorrhagic cystitis caused by adenoviruses 11 and 21. Epidemic keratoconjunctivitis, caused most frequently by adenovirus types 8, 19, and 37, has been associated with contaminated common sources such as ophthalmic solutions and roller towels.

### **3. Things to know about adenovirus biology and pertinent to your safety...**

The viruses constructed using this kit contain a deletion in the E1 region. E1A is the first transcription unit expressed during infection by adenovirus and the E1A proteins activate viral and cellular gene expression by multiple mechanisms. The net effect of E1A gene expression early after infection is a significant increase in the activity of the other early adenovirus promoter

regions, in particular the E2 region promoter which controls viral genes involved in DNA replication. E1A-deleted adenoviruses are therefore considered as replication-deficient. Their replication requires the expression of the E1A products *in trans*, such as in 293 cells. However, some cell lines can express factors that functionally compensate for the loss of E1A expression in adenovirus infection and allow the virus to replicate. For instance, HeLa cells, which express human papillomavirus (HPV) E6 and E7 regulatory proteins, whose functions are similar to those performed by Ad E1A and E1B 55-kDa proteins<sup>1</sup>, support replication of E1-deleted adenoviruses when infected with high virus titers.<sup>2</sup>

Ad5, the adenovirus strain at the origin of the vectors included in this kit, should pose no threat to humans. It has not been associated with malignancies in humans. However recent studies have shown that E1-substituted adenovirus vectors can integrate into the cellular chromosomes *in vitro* with efficiencies ranging from  $\sim 10^{-3}$  to  $10^{-5}$ .<sup>3</sup> Although no such study has been conducted *in vivo*, extreme caution should be exercised, considering the high titers of adenovirus you will probably end up working with.

The primary cellular receptor for adenovirus type 5 (CAR) has a wide tissue distribution. Adenovirus can therefore infect a large variety of dividing and non-dividing cells or tissues other than those targeted in the course of a natural infection. Extreme care should be taken when working with adenovirus, especially with high titers and when using needles.

#### **4. What facilities and equipment do you need to work with adenovirus?**

The National Institute of Health has designated adenoviruses as Level 2 biological agents. For most applications, working with adenovirus requires therefore a Biosafety Level 2 (BL2) facility. The NIH guidelines for research involving recombinant DNA molecules stipulate also that experiments which are likely to either enhance the pathogenicity (e.g. insertion of a host oncogene) or to extend the host range (e.g. introduction of novel control elements) of viral vectors under conditions that permit a productive infection should be performed in BL3 facilities.

A BL2 laboratory should contain:

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<sup>1</sup> Dyson N., Genes Dev. 1998 Aug 1;12(15):2245-62.

<sup>2</sup> O'Connor RJ, Hearing P, J Virol. 2000 Jul;74(13):5819-24.

<sup>3</sup> Harui et al., J Virol. 1999 Jul;73(7):6141-6.

- ◆ A warning sign on the entrance door limiting the access to authorized persons only. The sign should identify the agent, list the name and phone number of the lab director or other responsible person, and indicate any special requirement for entering the lab.
- ◆ A Class II biological safety cabinet. A Class II cabinet is a ventilated cabinet for personnel and product protection having an open front with inward airflow for personnel protection, and a HEPA filtered mass recirculated air flow for product protection. The face velocity of the inward flow of air through the full-width open front is 75 feet per minute or greater.
- ◆ At least one tissue culture incubator dedicated to infected cell cultures. Another separate incubator is desirable for growing uninfected cells.
- ◆ The minimal equipment to handle adenovirus culture without exiting the BL2 lab (such as centrifuges, microscope...).
- ◆ A sink for hand washing
- ◆ A chemical disinfectant kit or at least a gallon of bleach available for spills

## **5. What precautions should you take when working with adenovirus?**

- ◆ Always wear a lab coat while in the virus lab. Before exiting the laboratory for non-laboratory areas (e.g. cafeteria, library, administrative offices...), remove your lab coat and leave it in the laboratory.
- ◆ Avoid skin contamination with the virus. Always wear gloves (one pair OK, two pairs better for added protection). Once your gloves have been in contact with infectious material, do not touch common appliances such as telephone or doors handles. Change your gloves frequently.
- ◆ Keep the lab doors closed while work is in progress.
- ◆ Use mechanical pipetting devices. Do not pipet by mouth.
- ◆ Decontaminate all work surfaces after you finish your work, and immediately after any spill. Spray a 10% bleach solution, wipe and spray again a 70% ethanol solution. For large liquid spills, add directly concentrated bleach to the liquid, leave for at least 5 minutes, and wipe.
- ◆ Perform all procedures with infectious particles in the biosafety cabinet to minimize the exposure of personnel to aerosols. Minimize the creation of aerosols by pipetting virus cultures and suspension very gently. Use aerosol-resistant tips for pipetting virus suspensions. Do not

conduct work with infectious materials in open vessels on the open bench.

- ◆ Use needle-locking syringes or disposable syringe-needle units for the injection or aspiration of infectious fluids. Extreme care should be used to avoid auto-inoculation and aerosol generation. Needles should not be bent, sheared, replaced in their sheath or guard or removed from the syringe following use. The needle and syringe should be decontaminated by pipetting in and out concentrated bleach a few times and then promptly placed in a puncture-resistant container.
- ◆ Decontaminate all contaminated liquid or solid wastes before disposal. Before starting your virus work, pour some bleach into a beaker. Rinse all materials (tissue culture dishes, pipets, tips...) that came into contact with adenovirus with 10% bleach inside the hood before discarding them in the Biohazard trash and autoclaving. Place all materials to be decontaminated off-site in a durable leakproof container which is closed before removal. If possible, leave the contaminated materials in contact with bleach for a few hours before autoclaving (e.g. after rinsing your pipets with concentrated bleach inside the hood, soak them in a cylinder containing 10% bleach before autoclaving).
- ◆ Do not leave the BL2 laboratory with live viruses, unless they are in a sealed tube. Cell cultures transduced with adenoviruses should be inactivated either chemically or biochemically before leaving the BL2 facility.
- ◆ Store your adenovirus preparations at -70 °C in closed containers labeled with Biohazard warning signs.
- ◆ Wash your hands when exiting the laboratory.

## **6. What should you do in case of spill or accidental virus infection?**

- ◆ Treat large liquid spills immediately with concentrated bleach. Spray the surrounding zone with 10% bleach. Wipe and discard the wiping materials in the biohazard trash before autoclaving. Repeat with 70% ethanol.
- ◆ Place signs warning your coworkers about the spill and report immediately to the lab director.

## 7. More readings...

For further information about biosafety, we recommend reading the following publications from the NIH Division of Safety

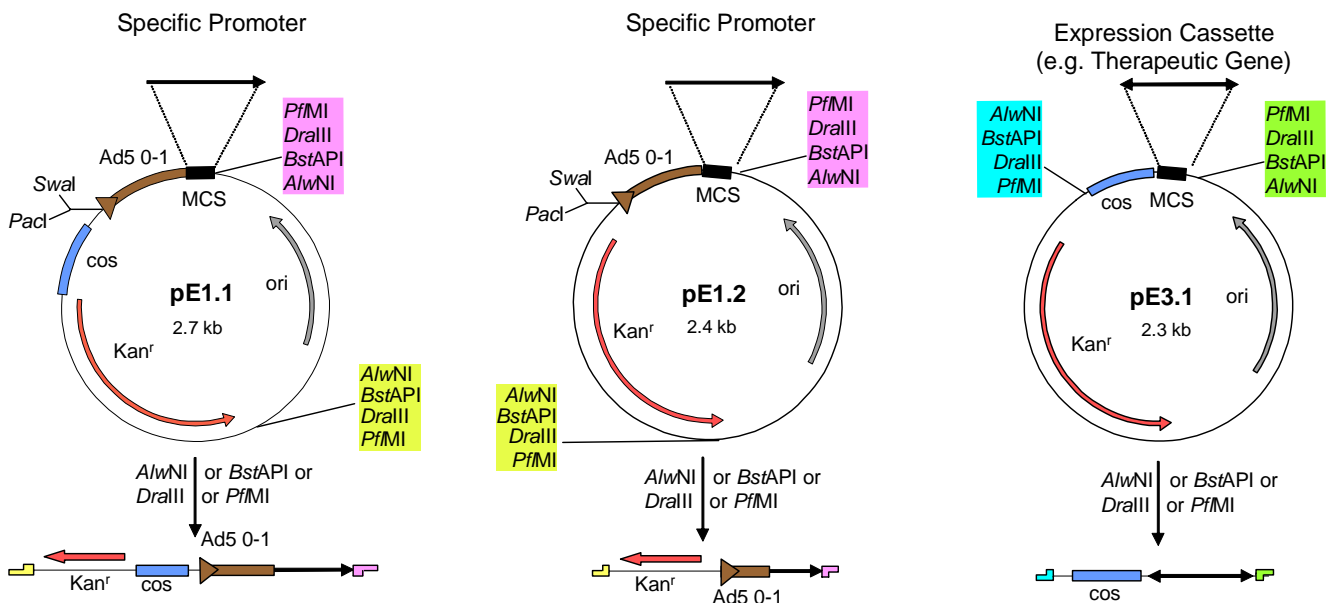
(<http://www.nih.gov/od/ors/ds/pubs/>):

- Biosafety in Microbiological and Biomedical Laboratories  
(<http://bmbf.od.nih.gov/>)
- NIH guidelines for research involving recombinant DNA molecules  
(<http://www4.od.nih.gov/oba/rdna.htm>)

Consult also your regional and institutional guidelines.

## C. Shuttle Plasmid Construction

The first step towards the construction of your conditionally replicative adenoviral vector is the insertion of your specific promoter or expression cassette into a small shuttle vector. The purpose of this cloning is to link these sequences to positive selection markers (kanamycin-resistance gene and/or  $\lambda$  *cos* site). This will facilitate the next step, i.e. the reconstitution of the entire genome of your recombinant virus in a plasmid or cosmid. Please refer to the table p. 9 to figure out which vectors you must use for your application.



**Figure 4: First Step towards the Construction of Your Recombinant CrAd.** A (tumor) specific promoter and/or expression cassette is inserted into the multiple cloning sites (MCS) of [pE1.1](#), [pE1.2](#) or [pE3.1](#). The resulting plasmids are then cut with PflMI, DraIII, BstAPI or A/wNI, whichever is not present in the expression cassette. The DNA fragment containing the expression cassette and the flanking sequences is purified on agarose gel. Kan<sup>r</sup>: Kanamycin-resistance gene; ori: pUC19 origin of replication; COS: lambda cos site; Ad5 0-1: map unit 0-1 (bp 1-353) of the Ad5 genome, including the left inverted terminal repeat and the packaging signal.

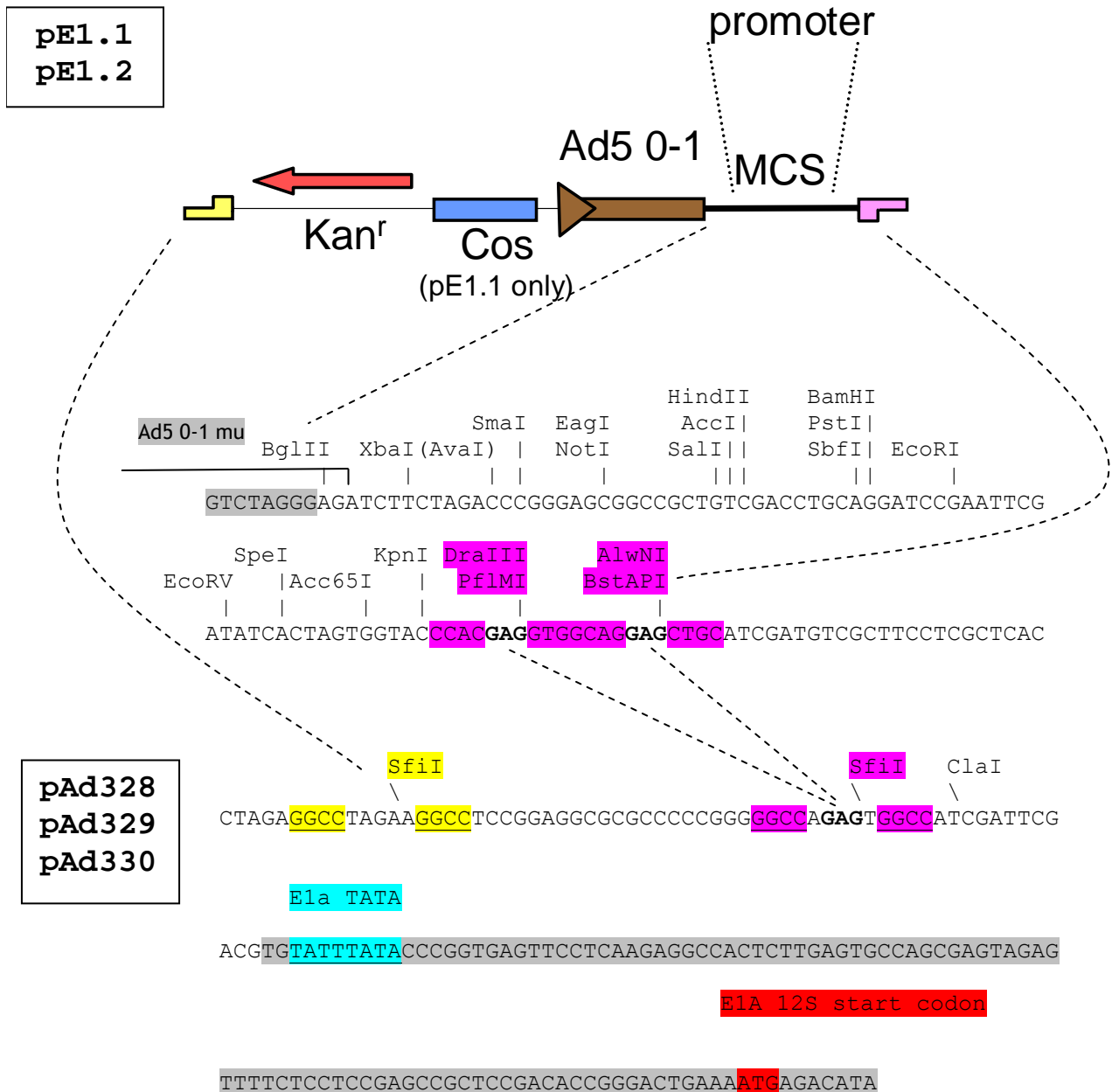
Plasmids pAd328, pAd329, and pAd330 contain the E1a TATA box, so that the promoter sequence that you intend to insert into pE1.1 or pE1.2 does not need to contain its own TATA box. The sequences surrounding the cloning sites in pE1.1 and pE1.2, and the E1a TATA box in pAd328, pAd329, and pAd330 are detailed on page 18.

If you plan to insert an expression cassette into the E3 region, be aware that the presence of an intron in that expression cassette might prevent virus recovery. Adenoviruses containing “CMV promoter/intron” expression cassettes in the E3 region have failed to be rescued. However viruses

expressing the same intron-containing cassette in the E1 region, and viruses expressing the same transgene under the control of an intron-less CMV promoter in the E3 region have been generated. The fact that the presence of a foreign intron in the E3 region prevents virus rescue might be caused by interference with the splicing of the 28 K mRNA transcribed from the major late promoter in the late phase of the virus cycle.

- Clone your expression cassette(s) into the polylinker of [pE1.1](#), [pE1.2](#), and [pE3.1](#), using established methods. The maps and polylinker sequences of these plasmids are detailed p. 38. Contact O.D.260 Inc in order to get the complete sequences of these plasmids.
- Digest the resulting plasmid either with *AlwNI*, *BstAPI*, *DraIII* or *PfI*MI (whichever is not present in your expression cassette), and purify the fragment containing your sequence of interest on agarose gel.
- ☺ *AlwNI*, *BstAPI*, *DraIII*, *PfI*MI and *Sfi*I are enzymes that recognize interrupted palindromes. The sequences that separate the two parts of the palindrome can be chosen arbitrarily. Upon cutting, they will all create 3 nt-long sticky ends with a 3' extension. In the AdenoQuick plasmids and their shuttle vectors pE1.1, pE1.2 and pE3.1, we have designed these sites in a specific way: there are 4 sets of *AlwNI*, *BstAPI*, *DraIII* and *PfI*MI sites (they are color-coded Figure 2 and Figure 3). Within one set, all four enzymes generate the same sticky end. For instance the *AlwNI*, *BstAPI*, *DraIII* and *PfI*MI sites in pE1.2, located downstream from the Kan-resistance gene (coded in yellow in Figure 3), all generate an AGA 3' extension as sticky end. This sticky end cannot be ligated with the sticky ends generated by the same enzymes at other places (such as the blue, green and pink sets shown in Figure 3). However this AGA sticky end can ligate with the sticky end generated by one of the *Sfi*I sites present in the AdenoQuick13.1 vector (also coded in yellow). It does not matter which enzyme you use to cut pE1.1, pE1.2 or pE3.1, as long as it does not cut inside your expression cassette. You can use the same enzyme or not for pE1.2 and pE3.1, the result will be almost exactly the same (a few nucleotides difference).

|   |                             |                                       |
|---|-----------------------------|---------------------------------------|
| ☺ | <b>Restriction enzymes:</b> | <a href="#">(New England Biolabs)</a> |
|   | <i>AlwNI</i>                | #R0514S                               |
|   | <i>BstAPI</i>               | #V0259S                               |
|   | <i>DraIII</i>               | #R0510S                               |
|   | <i>PfI</i> MI               | #R0509S                               |



**Figure 5:** Sequence of the pE1.1/pE1.2 multiple cloning site and the E1a region of pAd328, pAd329, and pAd330, highlighting the position of promoter insertion site versus the E1a TATA box. The promoter of interest should be cloned anywhere between the *XbaI* and *KpnI* sites of pE1.1 or pE1.2 shuttle vectors. The resulting plasmid will be cut with *DraIII*, *PflMI*, *AlwNI*, or *BstAPI* (whichever is not present in the promoter sequence), and the fragment of interest containing the Kanamycin-resistance gene, cos site (for pE1.1), Ad5 left ITR, and the promoter will be purified on agarose gel. The fragment will be ligated with *SfiI*-digested pAd328, pAd329, or pAd330. The restriction sites that produce compatible sticky ends are highlighted with the same color (yellow or purple). The E1a TATA box is indicated in blue, the start codon of the E1a 12S transcript is colored in red. All Ad5 sequences are shaded in grey.

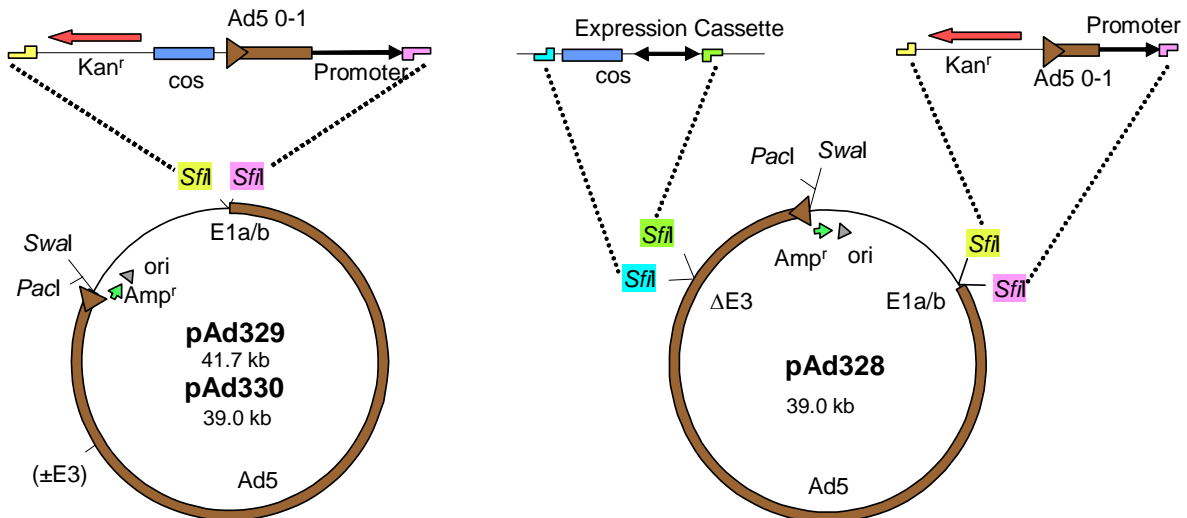
## D. Adenovirus Cosmid/Plasmid Construction

The second step towards the generation of your adenoviral vector is the reconstitution of the entire genome of your recombinant virus in a cosmid/plasmid.

Our system allows you to construct that cosmid/plasmid using two approaches:

1. [DNA packaging into phage  \$\lambda\$](#)  and infection of *E. coli*, or
2. direct transformation of *E. coli* by [electroporation](#).

The first method ( $\lambda$  packaging) is the most efficient. Since phage  $\lambda$  packages preferentially DNA molecules that are 39-54 kb long, it selects for clones that carry full-size genomes. This system is very efficient since practically 100% of the bacterial clones carry the correct cosmid. This allows experienced users to purify the cosmid DNA directly from a 50 mL bacterial culture even before verifying its identity. **We recommend strongly that you use that method to construct bipartite adenoviruses with pAd328.**



**Figure 6: Second Step towards the Construction of Your Recombinant CrAd.** The DNA fragment(s) containing the expression unit(s) and the positive selection markers ( $Kan^r$  and  $cos$  site) is (are) ligated with the *SfiI*-digested AdenoQuick plasmids and introduced into *E. coli* via DNA packaging into phage  $\lambda$  or by electroporation.  $Amp^r$ : Ampicillin-resistance gene;  $Kan^r$ : Kanamycin-resistance gene; ori: pUC19 origin of replication; COS: lambda cos site; Ad5 0-1: map unit 0-1 (bp 1-353) of the Ad5 genome, including the left inverted terminal repeat and the packaging signal.

Electroporation is less efficient (up to 70% correct clones for pAd329 and pAd330, about 10% for pAd328) and therefore you will have to analyze a series of clones before growing a larger culture. Both methods are comparable as far as the prices of reagents are concerned. The  $\lambda$  packaging method requires a little bit more hands-on time to set up, but is very easy. We recommend also that once you get the *E. coli* clones carrying your cosmid/plasmid, you grow immediately the mid- or large-size bacterial cultures. We do not advise to store the Petri plates at 4 °C for a few days before growing the large-scale cultures, since in some cases it is difficult to recover the cosmid.

## Option 1: Adenovirus Cosmid Construction via DNA Packaging into Phage $\lambda$

### DAY 1

- Ligate 2  $\mu$ L *Sfi*I-digested [pAd328](#), [pAd329](#) or [pAd330](#) (about 400-500 ng) with 400 ng of insert(s) in a 10  $\mu$ L volume, for 1 hour at room temperature to overnight at 16°C.

|                          | Specific promoter in front of E1 region |                                | Specific promoter in front of E1 region+ expression cassette in E3 region |
|--------------------------|---|--------------------------------|---|
|                          | WT E3                                   | $\Delta$ E3                    |   |
| AdenoQuick plasmid       | 2 $\mu$ L pAd329/ <i>Sfi</i> I          | 2 $\mu$ L pAd330/ <i>Sfi</i> I | 2 $\mu$ L pAd328/ <i>Sfi</i> I  |
| Insert E1                | x $\mu$ L                               | x $\mu$ L                      | x $\mu$ L   |
| Insert E3                | N/A                                     | N/A                            | y $\mu$ L   |
| H <sub>2</sub> O         | 6-x $\mu$ L                             | 6-x $\mu$ L                    | 6-x-y $\mu$ L   |
| 10x T4 DNA Ligase buffer | 1 $\mu$ L                               | 1 $\mu$ L                      | 1 $\mu$ L   |
| T4 DNA Ligase            | 1 $\mu$ L                               | 1 $\mu$ L                      | 1 $\mu$ L   |
| <b>Total volume</b>      | <b>10 <math>\mu</math>L</b>             | <b>10 <math>\mu</math>L</b>    | <b>10 <math>\mu</math>L</b>   |

- ☺ pAd328, pAd329, and pAd330 DNAs are provided pre-digested with *Sfi*I. They are shipped in TE<sup>4</sup> pH 7.5 at room temperature. Before opening the vial, centrifuge it briefly to pellet the contents to the bottom of the tube.
- ☺ Always check the quality and the fluorescence intensity of the purified DNA fragments on agarose gel. This will help you estimating the amounts of DNA to

<sup>4</sup> TE: 10 mM Tris, 1 mM EDTA

ligate. Make sure that you ligate a 3x to 6x molar excess of insert compared to the vector. Working with too small DNA amounts will lower the overall cloning efficiency.

- ☺ T4 DNA Ligase: 400 units/ $\mu$ L (NEB # M0202)
  
- ☐ Streak *E. coli* from a glycerol stock on a LB plate. Incubate overnight at 37 °C.
  
- ☺ XL1-blue ([Stratagene](#)) and Top 10 ([Invitrogen](#)) work well: cosmids are stable and DNA yields are high. DH5 $\alpha$  is somewhat less efficient.

## DAY 2

### First thing in the morning:

- ☐ Prepare 2 mL LB supplemented with 0.2% maltose and 10 mM MgSO<sub>4</sub>.
  - ☺ Sterile 10% Maltose and 1 M MgSO<sub>4</sub> solutions are provided in the AdenoQuick kits.
  
- ☐ Inoculate with one *E. coli* colony.
- ☐ Grow at 37 °C for 4-6 hours until the culture appears dense. Do not overgrow. Chill the cells on ice, and measure the cell density by absorbance at 600 nm (OD<sub>600</sub>).

**Packaging:** The following procedure uses the MaxPlax Lambda packaging extract from Epicentre, which works very well.

- ☐ Add 5  $\mu$ L ligation mixture to a microcentrifuge tube.
- ☐ Thaw a vial of  $\lambda$  packaging extract, keeping it on ice as much as possible. Once thawed, add immediately 15  $\mu$ L packaging extract to the ligation mixture. Mix by pipetting in/out once. Avoid bubbles and do not spin down.
- ☐ Incubate the extract at 30 °C for 90 min.
- ☐ Add 250  $\mu$ L SM buffer (provided in the kits) and 12.5  $\mu$ L chloroform. Mix vigorously for a few seconds with your fingertips. White debris will appear. Centrifuge for a few seconds, transfer the supernatant to a new tube (avoid the chloroform) and keep on ice or at 4 °C.

- ☺ MaxPlax Lambda packaging extract: Epicentre cat# MP5105. The packaging extracts Gigapack III Plus, Gigapack III XL and Gigapack III Gold from [Stratagene](#) (cat # 200201, 200204, 200207) work also well but are more expensive.
- ☺ You can reduce the amount of packaging extract for the reaction but make sure to keep a ratio 1:2.5 between ligation mixture and packaging extract. We have successfully constructed cosmids using 2  $\mu$ L ligation mixture and 5  $\mu$ L packaging extract. If you do so, adjust the volumes of SM buffer and chloroform accordingly.
- ☺ You can save the remainder of the packaging extract for later use: immediately after pipetting the extract from the tube, quickly freeze the remainder in liquid N<sub>2</sub>. The efficiency of the next reaction will not be significantly reduced.
- ☺ The packaged phage extract is stable for a few months at 4°C.
- ☺ SM buffer (100 mL):
 

|             |                      |
|-------------|----------------------|
| 2 mL        | 5M NaCl              |
| 800 $\mu$ L | 1M MgSO <sub>4</sub> |
| 5 mL        | 1M Tris pH7.5        |
| 0.5 mL      | 2% (w/v) gelatin     |
| 92 mL       | H <sub>2</sub> O     |

### Infection:

- ☐ Dilute the competent bacteria to OD<sub>600</sub> = 1.0 in LB. Mix 50  $\mu$ L packaging mixture with 50  $\mu$ L of the diluted cells in a microcentrifuge tube and incubate at 37°C for 30 min. Avoid bubbles and do not spin down.
- ☐ Spread the bacteria on a LB plate supplemented with 50  $\mu$ g/mL ampicillin and 25  $\mu$ g/mL kanamycin. Incubate overnight at 37 °C.
- ☺ In presence of kanamycin, Top10 bacteria grow faster in low salt medium (Lennox: 5 g/L NaCl) than high salt medium (Luria Bertani, Miller: 10 g/L NaCl).

### DAY 3

- ☐ Start early! Inoculate 2 mL LB Lennox + 50  $\mu$ g/mL ampicillin and 25  $\mu$ g/mL kanamycin with several clones. When approaching saturation, prepare the plasmid DNA and verify the identity of the cosmid by restriction analysis.
- ☺ The cloning efficiency of this system is very high. It is practically 100% for the constructions involving the ligation of 2 fragments (pAd329 and pAd330). It is more than 90% for the constructions involving the ligation of 4 fragments (pAd328). Therefore you need to analyze only 2-4 clones per construction.

- ☺ Use your favorite method for plasmid purification. The standard alkaline lysis method works fine. We have used the Wizard Plus SV Minipreps DNA Purification System (Promega #A1460). This kit yields good-quality DNA that can be used directly to recover the virus by transfection into 293 cells.
- ☐ Inoculate one of the correct clones into 50 mL LB supplemented with 50 µg/mL ampicillin and 25 µg/mL kanamycin. Monitor the cell density by measuring the absorbance at 600 nm. Harvest the cells at the end of the exponential growth phase, i.e. when OD<sub>600</sub> is between 2.0 and 2.5 (usually after 8-10 hours).
- ☺ Top10 bacteria grow more slowly in presence of kanamycin than in presence of ampicillin but the DNA yield is higher. This is not the case with XL-1 blue.
- ☺ It is important not to let the cells grow for a too long period of time, since DNA yield will drop and DNA recombinations might occur. Do not grow for more than 12 hours.
- ☺ A 50-mL culture should provide enough DNA for the virus recovery step.

#### DAY 4

- ☐ Purify the cosmid DNA using the alkaline lysis method followed by a purification step of your choice. We have used double CsCl gradient, Nucleobond midi-columns ([Clontech](#)), and Wizard Purefection Plasmid prep midi kit ([Promega](#)). All methods yielded DNA that was able to generate virus. Our favorite is the Promega Wizard kit.
- ☺ It is important to have a DNA as pure as possible, since its quality will affect the transfection efficiency and virus recovery. Avoid genomic DNA.
- ☺ Remember not to dry the cosmid too much after ethanol or isopropanol precipitation. Pipet cosmid solutions gently in order not to shear the DNA.
- ☐ Verify the integrity of your cosmid by restriction analysis.
- ☐ Digest 20 µg cosmid with either *PacI* or *SwaI*, whichever is not present in your gene of interest. After digestion, do not purify the DNA on agarose gel. Do not phenolize, but precipitate the DNA directly with EtOH. Resuspend the DNA in sterile TE pH 7.5 at a concentration of 0.5 µg/µL.
- ☺ The AdenoQuick system offers the choice between *PacI* and *SwaI* for excising the adenovirus genome from the plasmid. Both enzymes are 8-base cutters,

thus they should be present at equal frequencies in DNA. The only difference is the position of the restriction sites relative to the start of the adenovirus ITR. As illustrated below, *PacI* and *SwaI* generate 3 and 11 nt-long hanging sequences, respectively:



The adenoviruses that will be generated from either *PacI* or *SwaI*-linearized DNAs will be identical: their genome will start with the correct nucleotide sequence (as highlighted in green). Indeed, the replication of adenovirus DNA is a protein-primed mechanism where an intermediate, the pre-terminal protein covalently linked to the first three nucleotides CAT, is synthesized opposite to positions 4-6 (underlined) before jumping back to position 1 of the template to start elongation.<sup>5</sup>

The DNA ends generated by *PacI* resemble the most the ends obtained from deproteinised virion DNA and might therefore be more efficient in promoting virus replication. In practice however, no difference in the time needed to recover the virus after DNA transfection into 293 cells is observed between both settings. Virus plaques can appear as early as 4 days after transfecting *PacI*- or *SwaI*-digested DNA into 293 cells.

☺ It is very important to work with clean materials and reagents: for instance, a trace of exonuclease could destroy the origins of viral DNA replication, which are close to the DNA ends, and prevent the DNA from generating virus plaques after transfection into helper cells.

- Proceed to the DNA transfection into helper cells for [virus recovery](#) (p. 29).

<sup>5</sup> King *et al*, EMBO J. 1994 Dec 1;13(23):5786-92.

## Option 2: Adenovirus Plasmid Construction via Electroporation

### DAY 1

- Ligate 2  $\mu\text{L}$  *Sfi*I-digested [pAd328](#), [pAd329](#) or [pAd330](#) (about 400-500 ng) with 400 ng of insert(s) in a 10  $\mu\text{L}$  volume, for 1 hour at room temperature to overnight at 16°C.

|                          | Specific promoter in front of E1 region |                                      | Specific promoter in front of E1 region+ expression cassette in E3 region |
|--------------------------|---|--------------------------------------|---|
|                          | WT E3                                   | $\Delta$ E3                          |   |
| AdenoQuick plasmid       | 2 $\mu\text{L}$ pAd329/ <i>Sfi</i> I    | 2 $\mu\text{L}$ pAd330/ <i>Sfi</i> I | 2 $\mu\text{L}$ pAd328/ <i>Sfi</i> I                                      |
| Insert E1                | x $\mu\text{L}$                         | x $\mu\text{L}$                      | x $\mu\text{L}$   |
| Insert E3                | N/A                                     | N/A                                  | y $\mu\text{L}$   |
| H <sub>2</sub> O         | 6-x $\mu\text{L}$                       | 6-x $\mu\text{L}$                    | 6-x-y $\mu\text{L}$   |
| 10x T4 DNA Ligase buffer | 1 $\mu\text{L}$                         | 1 $\mu\text{L}$                      | 1 $\mu\text{L}$   |
| T4 DNA Ligase            | 1 $\mu\text{L}$                         | 1 $\mu\text{L}$                      | 1 $\mu\text{L}$   |
| <b>Total volume</b>      | <b>10 <math>\mu\text{L}</math></b>      | <b>10 <math>\mu\text{L}</math></b>   | <b>10 <math>\mu\text{L}</math></b>  |

- ☺ pAd328, pAd329, and pAd330 DNAs are provided pre-digested with *Sfi*I. They are shipped in TE<sup>6</sup> pH 7.5 at room temperature. Before opening the vial, centrifuge it briefly to pellet the contents to the bottom of the tube.
- ☺ Always check the quality and the fluorescence intensity of the purified DNA fragments on agarose gel. This will help you estimating the amounts of DNA to ligate. Make sure that you ligate a 3x to 6x molar excess of insert compared to the vector. Working with too small DNA amounts will lower the overall cloning efficiency.
- ☺ T4 DNA Ligase: 400 units/ $\mu\text{L}$  (NEB # M0202)

### DAY 2

- Desalt the ligation reaction: apply the ligation mixture carefully on a MF-Millipore Membrane Filter ([Millipore](#) cat # VSWP02500) floating on distilled water in a Petri dish (shiny side up) and let the salts diffuse for 20 min.

<sup>6</sup> TE: 10 mM Tris, 1 mM EDTA

- The conditions for electroporation may vary from machine to machine. We use a [BTX](#) Electro Cell Manipulator 600, or Biorad Gene Pulser II with 2 mm-gap electroporation cuvettes from BTX or Invitrogen. Electro-competent *E. coli* Top10 are prepared according to the BTX protocol for *E. coli* DH5 $\alpha$ .
- Add 1  $\mu$ L desalted ligation mix to 40  $\mu$ L freshly thawed electro-competent Top10 cells. Transfer to a chilled electroporation cuvette.
- Apply an electric shock as recommended by the manufacturer of your machine: 2.45 kV, 129 Ohms (BTX); 2.5 kV, 200 Ohms, 25  $\mu$ F (Biorad).
- Immediately after the electric shock, add 400  $\mu$ L LB or SOC medium, and shake at 37 °C for 45 min.
- Spread the bacteria on LB dishes supplemented with 50  $\mu$ g/mL ampicillin and 25  $\mu$ g/mL kanamycin.

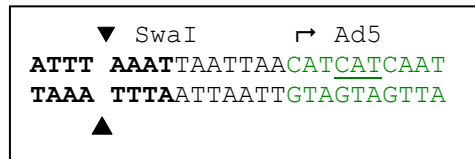
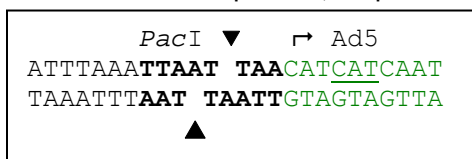
### DAY 3

- Start early: inoculate 10-12 clones in 2 mL LB supplemented with 50  $\mu$ g/mL ampicillin and 25  $\mu$ g/mL kanamycin.
- When approaching saturation, prepare the plasmid DNAs and verify the identity of the plasmids by restriction analysis.
  - ☺ Use your favorite method for plasmid purification. The standard alkaline lysis method works fine. We have used the Wizard Plus SV Minipreps DNA Purification System (Promega #A1460). This kit yields good-quality DNA that can be used directly to recover the virus by transfection into 293 cells.
- Inoculate one of the correct clones into 50 mL LB supplemented with 50  $\mu$ g/mL ampicillin and 25  $\mu$ g/mL kanamycin. Monitor the cell density by measuring the absorbance at 600 nm. Harvest the cells at the end of the exponential growth phase, i.e. when OD<sub>600</sub> is between 2.0 and 2.5 (usually after 8-10 hours).
  - ☺ Top10 bacteria grow more slowly in presence of kanamycin than in presence of ampicillin but the DNA yield is higher. This is not the case with XL-1 blue.
  - ☺ It is important not to let the cells grow for a too long period of time, since DNA yield will drop and DNA recombinations can occur. Do not grow for more than 12 hours.

- ☺ A 50-mL culture should provide enough DNA for the virus recovery step.

## DAY 4

- Purify the plasmid DNA using the alkaline lysis method followed by a purification step of your choice. We have used double CsCl gradient, Nucleobond midi-columns ([Clontech](#)), and Wizard Purefection Plasmid prep midi kit ([Promega](#)). All methods yielded DNA that was able to generate virus. Our favorite is the Promega Wizard kit.
- ☺ Using Top10 and the Promega DNA purification kit, an average of 1 µg DNA/mL bacterial culture is usually obtained.
- ☺ It is important to have a DNA as pure as possible, since its quality will affect the transfection efficiency and virus recovery.
- ☺ Remember not to dry the large plasmid too much after ethanol or isopropanol precipitation. Pipet large plasmid solutions gently in order not to shear the DNA.
- Verify the integrity of your plasmid by restriction analysis.
- Digest 20 µg plasmid with either *PacI* or *SwaI*, whichever is not present in your gene of interest. After digestion, do not phenolize, but precipitate the DNA directly with EtOH. Resuspend the DNA in sterile TE pH 7.5 at a concentration of 0.5 µg/µL.
- ☺ The AdenoQuick system offers the choice between *PacI* and *SwaI* for excising the adenovirus genome from the plasmid. Both enzymes are 8-base cutters, thus they should be present at equal frequencies in DNA. The only difference is the position of the restriction sites relative to the start of the adenovirus left ITR. As illustrated below, *PacI* and *SwaI* generate 3 and 11 nt-long hanging sequences, respectively:



The adenoviruses that will be generated from either *PacI* or *SwaI*-linearized DNAs will be identical: their genome will start with the correct nucleotide sequence (as highlighted in green). Indeed, the replication of adenovirus DNA is a protein-primed mechanism where an intermediate: the pre-terminal protein covalently linked to the first three nucleotides CAT, is synthesized

opposite to positions 4-6 (underlined) before jumping back to position 1 of the template to start elongation.

The DNA ends generated by *PacI* resemble the most the ends obtained from deproteinised virion DNA and might therefore be more efficient in promoting virus replication. In practice however, no difference in the time needed to recover the virus after DNA transfection into 293 cells is observed between both settings. Virus plaques can appear as early as 4 days after transfecting *PacI*- or *SmaI*-digested DNA into 293 cells.

- ☺ It is very important to work with clean materials and reagents: for instance, a trace of exonuclease could destroy the origins of viral DNA replication, which are close to the DNA ends, and prevent the DNA from generating virus plaques after transfection into helper cells.
- Proceed to the DNA transfection into helper cells for [virus recovery](#) (p. 29).

## E. Virus Recovery

The next step towards the construction of your recombinant adenovirus is the transfection of the viral DNA into helper cells. Helper cells stably express the products of the adenovirus E1 region and therefore complement for the absence of this region in your recombinant vector.

The most common helper cells for first generation adenoviruses are Ad5-transformed human embryonic kidney (HEK) 293 cells. These cells can be transfected very easily using the calcium-phosphate/DNA precipitation technique.

Because of the entire sequence of your recombinant virus was reconstituted in a cosmid or large plasmid, and the plasmid was purified from a single *E. coli* clone, a homogeneous virus population should be generated upon transfection of the helper cells.

However we recommend performing at least one plaque assay to isolate viral clones. The main reason is linked to the stability of your recombinant virus. This latter might not be stable if, for instance, it expresses a protein toxic for the helper cells, or a product that interferes with the viral replication cycle, or if the length of your expression cassette exceeds the maximal transgene capacity of the virus. Primarily because of the first two reasons, it is difficult to predict whether your recombinant virus will be stable. It is therefore good virological practice to isolate and analyze several virus plaques.\*

Viral crude extracts obtained directly from the dishes transfected with the DNA ligation mixtures may be used in preliminary experiments, e.g. to verify transgene expression from your recombinant virus, especially before starting clone purification and large-scale virus preparation.

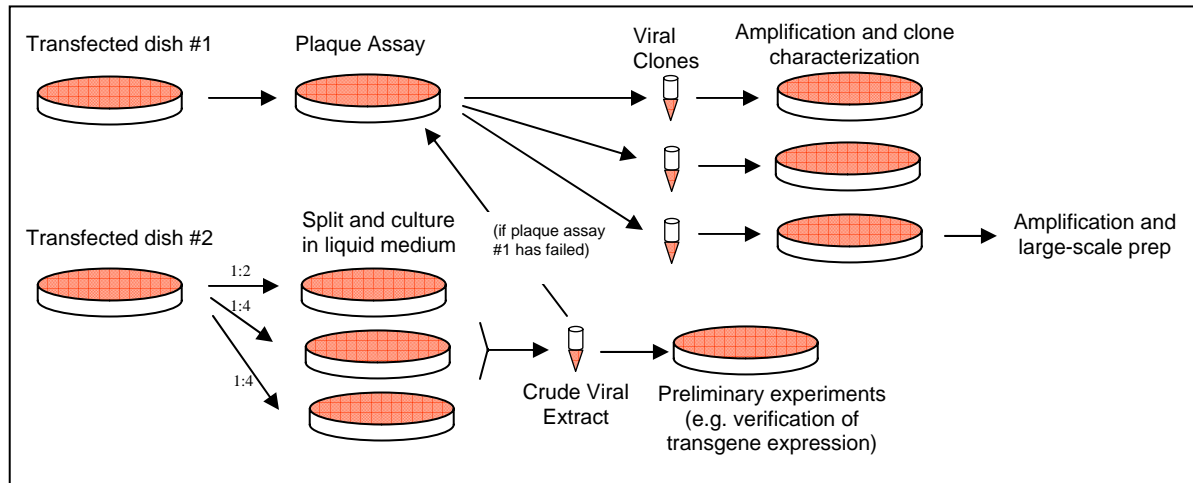
In order to maximize your chances of recovering your recombinant virus quickly, we propose you to follow the procedure described in Figure 7.

Briefly, two 293 cell dishes per construct are transfected with the *PacI*- or *SwaI*-linearized cosmid/plasmid DNA. The first dish is overlaid with agar. Three plaques are harvested, amplified and analyzed for transgene expression and genome stability. The second dish is kept under liquid medium, and split 2 or 3 days after the transfection, a procedure which will boost the appearance

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\* Please note that for the same reasons, plaque assays should be performed with any method based on the reconstitution of the entire sequence of the recombinant virus in a plasmid or cosmid in *E. coli*.

of virus plaques. This second dish is harvested when the entire cell monolayer has undergone a cytopathic effect. This crude viral extract will serve as back-up in case the plaque assay performed with the first dish has failed, and can be used for a quick verification of transgene expression.



**Figure 7:** Proposed Flow-Chart to Isolate, Analyze and Amplify your Recombinant Virus.

## a. Cell culture

- About one week before the expected transfection day, start culturing the 293 cells. Quickly thaw a vial of frozen 293 cells in a 37°C water bath and transfer the cells to a 10-cm dish containing 10 mL warm DMEM supplemented with 10% FBS and antibiotic/antimycotic reagent. Change the medium the next day. If necessary, split the cells 1:4 as soon as they reach confluence.
  - ☺ Use low-passage 293 cells (ATCC CRL-1573): this will facilitate the recovery of your recombinant virus, increase your virus yields and shorten the duration of the plaque assays. Cells up to passage 45 work well.
  - ☺ Use fresh cell culture media:
    - DMEM (high glucose, with L-glutamine)
    - Fetal Bovine Serum (not heat inactivated)
    - Trypsin-EDTA
    - Penicillin/Streptomycin
- On the day before the transfection, split a newly confluent 10-cm dish of 293 cells into six 6-cm dishes (DMEM supplemented with 10% FBS and

antibiotic/antimycotic agent). This is best performed in the late afternoon. The next morning, the cells should be 80-90 % confluent.

- ☺ Plan to transfect 2 dishes per adenovirus construct. Include also two negative controls (untransfected cells) and, if you deem it necessary, a positive control for the transfection. This latter control consists in transfecting 293 cells with a cosmid-purified adenovirus DNA expressing *E. coli*  $\beta$ -galactosidase (AdBGal cat # ZC-01). This  $\beta$ -Gal virus should be useful in your future experiments.

## b. Transfection

- ☺ HEK 293 cells can be transfected very easily using the calcium-phosphate/DNA precipitation technique. The following recipe can yield up to 30 plaques/ $\mu$ g DNA.

- Prepare 2x HBS and CaCl<sub>2</sub> 2M:

2x HBS (25 mL):     Add   - 1.4 mL NaCl 5 M  
                                      - 2.5 mL Hepes 0.5 M  
                                      - 37.5  $\mu$ L Na<sub>2</sub>HPO<sub>4</sub> (or NaH<sub>2</sub>PO<sub>4</sub>) 1 M  
                                      - H<sub>2</sub>O to 22 mL  
                                      Adjust to pH 7.0  
                                      Adjust volume to 25 mL with H<sub>2</sub>O  
                                      Filter sterilize

- ☺ The pH of the HBS is very important. We recommend strongly preparing fresh 2x HBS each time you perform a transfection instead of freezing aliquots. This should take no more than 15 minutes and it will maximize your chances of recovering the virus quickly.

- In a sterile microcentrifuge tube, mix in the following order:

| <i>Reagent</i>       | <i>Your Recombinant virus<br/>(Optional: in duplicate)</i> | <i><math>\beta</math>-Gal control virus<br/>(Optional)</i> |
|----------------------|--|--|
| H <sub>2</sub> O     | 199 $\mu$ L  | 215 $\mu$ L  |
| CaCl <sub>2</sub> 2M | 31 $\mu$ L   | 31 $\mu$ L   |
| DNA                  | 20 $\mu$ L   | 4 $\mu$ L (2 $\mu$ g)                                      |
| <b>Total volume</b>  | <b>250 <math>\mu</math>L</b>                               | <b>250 <math>\mu</math>L</b>                               |

- In a 5-mL round-bottom polypropylene tube (e.g. Falcon, Becton-Dickinson # 35-2063), dispense 250  $\mu$ L 2x HBS. Slowly add the H<sub>2</sub>O/CaCl<sub>2</sub>/DNA solution, drop-wise, mixing gently and continuously. Wait for 30 sec. Sprinkle onto

the cells using 1 mL Pipetman. Do not swirl, put the cells straight back into the incubator.

- Approximately eight hours after the transfection, rinse the cells twice with DMEM/10 % FBS.
  - ☺ This step is important to ensure healthy cell growth and virus recovery. In some cases, cells that were not washed will acquire a necrotic phenotype, which can be mistakenly identified as a cytopathic effect caused by the virus.

### c. Virus Clone Isolation

*One or two days after transfection, the cell monolayers of the 2 transfected dishes should reach confluence.*

#### Transfected Dish #1

- Overlay the cell monolayer of one transfected dish with agar noble: remove the medium from the dish, and overlay with 10 mL agar mixture (standard plaque assay protocol - do not incorporate neutral red). If necessary, perform a second agar overlay (5 mL) six days later to feed the cells.
  - ☺ Do not overlay all the transfected dishes with agar, since sometimes the cell monolayers do not survive the agar overlay. Plaques appear on average 7-10 days after transfection, sometimes as early as 3 days after transfection. This duration depends on several parameters such as the quality of the cells, the efficiency of transfection, and the nature of your expression cassette.
- When plaques are 2-3 mm in diameter, pick three of them with a large-bored aerosol-resistant 1-mL tip and resuspend them in 500  $\mu$ L DMEM/10% FBS in a sterile microcentrifuge tube. Freeze/thaw 3 times to release the virus from the cells. Spin down the agar and the cell debris (30 sec. 12,000 rpm). Transfer half the supernatants to cryovials and store at -70°C.
- With the other half, infect 293 cell monolayers seeded in 6-cm dishes: remove the culture medium and add directly 250  $\mu$ L virus extract supplemented with 250  $\mu$ L DMEM/10% FBS (total = 500  $\mu$ L). Incubate the cultures at 37 °C and swirl the dishes every 15 min. for 1 h (in two orthogonal directions to ensure that the whole monolayer is covered). Add an additional 4 mL DMEM/10% FBS and incubate at 37 °C.
- Harvest the cells and medium when >90% of the cells have detached from the dish. Split equally into two 15-mL sterile polypropylene tubes.

- Freeze/thaw the first tube 3 times in order to release the virus from the cells. Spin down the cell debris by centrifugation (400 g, 5 min.). Transfer the supernatants to cryovials and store at -70°C. This crude viral extract can be used to verify transgene expression in a reporter cell line and amplify the virus further.
- Spin the second tube for 5 min. at 400 g (1200 rpm) in a table-top centrifuge. Remove the supernatant, break the cell pellet by flicking the tube with your finger, add 1 mL PBS, and transfer the cell suspension to a 1.5 mL centrifuge tube. Spin for 5 min at 3000 rpm in a mini-centrifuge. Discard the supernatant. The cell pellet can be frozen at this point. It will be used to extract the viral genomic DNA using the Hirt method, and characterize your recombinant virus by restriction analysis, PCR, or Southern. By comparing the restriction patterns of the 3 virus isolates, you will be able to assess the stability of your construct.

## Transfected Dish #2

- Three days after the transfection, split the second transfected dish into one 10-cm dish.
  - ☺ Adenovirus is unstable in acidic medium, and replicates better in “healthy” dividing cells. Splitting the transfected cells should boost the virus recovery. Rounded cells should appear soon, first attached to the plate, and then floating. In some cases, the cell monolayer will probably not reach 100% confluence and therefore plaques will be difficult to spot.
- When the presence of virus is evident (i.e. more and more rounded and floating cells, with less and less attached, elongated cells on the bottom of the dish), harvest the entire dish (cells + medium) and split equally into two 15-mL sterile polypropylene tubes.
- Freeze/thaw the first tube 3 times in order to release the virus from the cells. Spin down the cell debris by centrifugation (1200 rpm 5 min.). Transfer the supernatants to cryovials and store at -70°C. This crude viral extract can be used to verify transgene expression in a reporter cell line and serve as a back up in case the plaque assay performed with dish #1 failed.
- Spin the second tube for 5 min. at 1200 rpm (400 g) in a table-top centrifuge. Remove the supernatant, break the cell pellet by flicking the tube with your fingers, add 1 mL PBS, and transfer the cell suspension to a

1.5 mL centrifuge tube. Spin for 5 min at 3000 rpm in a mini-centrifuge. Discard the supernatant in 10% bleach. The cell pellet can be frozen at this point. It will be used to extract the viral genomic DNA using the Hirt method<sup>7</sup>, and characterize your recombinant virus by restriction analysis.

- If the cell monolayers become very dense or the medium becomes acidic (yellow) without any apparent cytopathic effect (i.e. no more than five days after splitting), split the dishes again (1:2) and culture the cells until virus plaques appear, for up to 2 weeks. Feed the cells every 3-4 days with ¼ volume DMEM/10% FBS

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<sup>7</sup> Hirt B. (1967). J. Mol Biol 26:365-369.

## F. Plasmid Maps

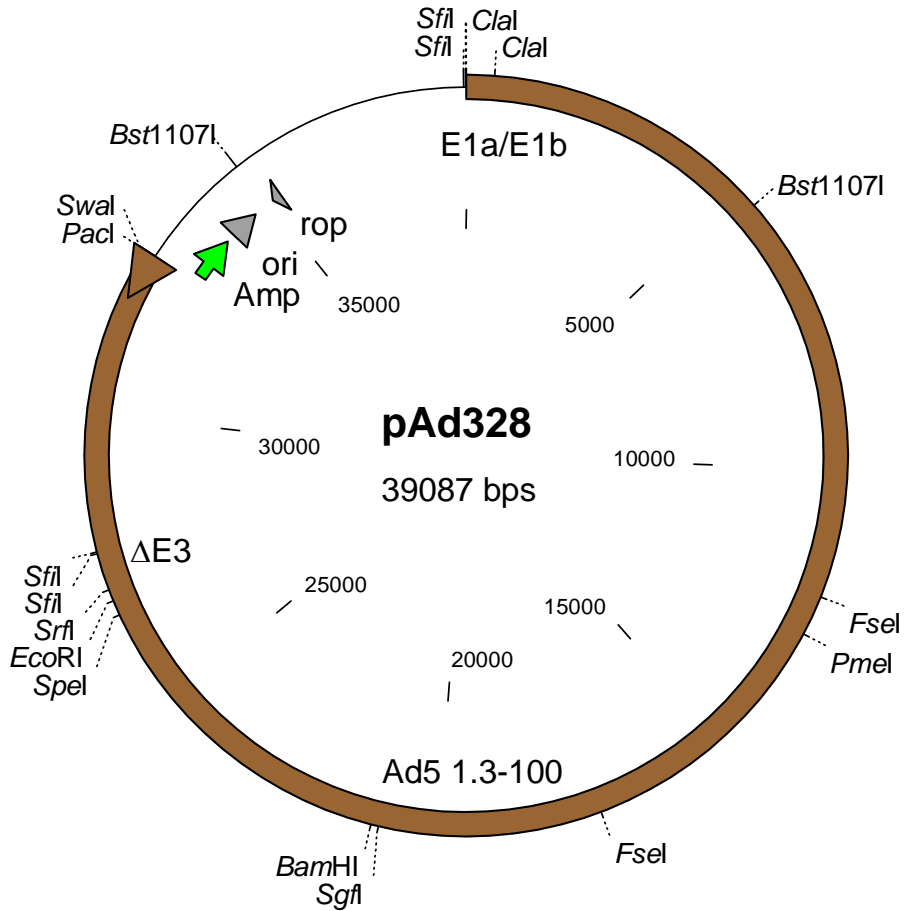
### pAd328

39,087 base pairs

Sequence available at [www.od260.com](http://www.od260.com)

pAd328 is a 39 kb plasmid that contains the sequences encompassing bp 466-right end (1.3-100 mu) of the Ad5 genome. The two *SfiI* sites naturally present in WT Ad5 DNA were mutated by substituting A for G and C at positions 16291 and 16294 in the Ad5 genome, and C and G for respectively G and C at positions 23001 and 23004 in the Ad5 genome, introducing silent mutations in the adenovirus pVII and DNA-binding protein coding sequences. Two pairs of *SfiI* sites that allow for directional cloning replace the E1 promoter and the E3 region. The size of the E3 region deletion is 2.7 kb. The right ITR is flanked by *PacI* and *SwaI* sites. pAd328 is used in combination with the shuttle vectors pE1.2 and pE3.1 to construct conditionally replicative adenoviruses (CrAds) containing a heterologous promoter in front of the E1a TATA box, and a transgene in place of the E3 region. The maximum combined promoter/transgene capacity is 4.6 kb.

| Feature               | Coordinates   | Source     |
|-----------------------|---------------|------------|
| 1.3-100 mu            | 13-32,863     | Ad5        |
| Right ITR             | 32,762-32,863 | Ad5        |
| origin of replication | 34,045-34,633 | pUC19      |
| Amp <sup>r</sup>      | 33,015-33,874 | <i>Tn3</i> |



Single and double cutters only.

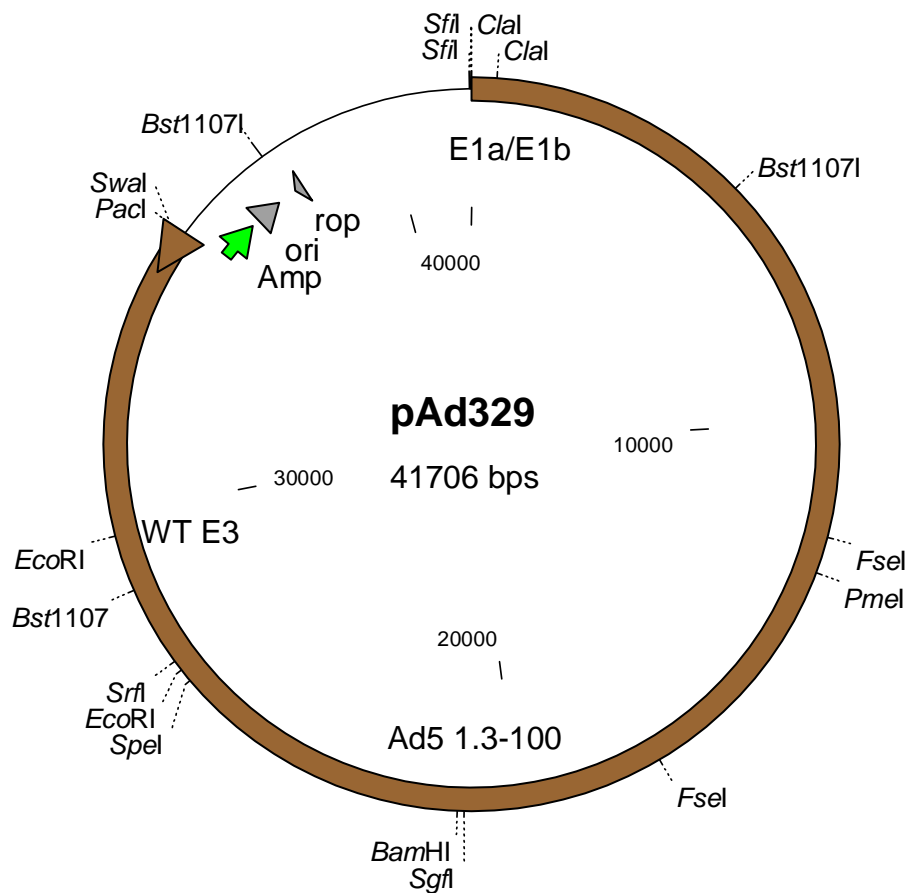
# pAd329

41,706 base pairs

Sequence available at [www.od260.com](http://www.od260.com)

pAd329 is a 41.7 kb plasmid that contains the sequences encompassing bp 466-right end (1.3-100 mu) of the Ad5 genome. The two *SfiI* sites naturally present in WT Ad5 DNA were mutated by substituting A for G and C at positions 16291 and 16294 in the Ad5 genome, and C and G for respectively G and C at positions 23001 and 23004 in the Ad5 genome, introducing silent mutations in the adenovirus pVII and DNA-binding protein coding sequences. A pair of *SfiI* sites that allow for directional cloning replaces the E1 promoter. The E3 region is intact. The right ITR is flanked by *PacI* and *SwaI* sites. pAd329 is used in combination with the shuttle vector pE1.1 to construct conditionally replicative adenoviruses (CrAds) containing a heterologous promoter in front of the E1a TATA box. The maximum capacity for insertion is 2.1 kb.

| Feature               | Coordinates   | Source     |
|-----------------------|---------------|------------|
| 1.3-100 mu            | 13-35,482     | Ad5        |
| Right ITR             | 35,380-35,482 | Ad5        |
| origin of replication | 36,664-37,252 | pUC19      |
| Amp <sup>r</sup>      | 35,634-36,493 | <i>Tn3</i> |



Single and double cutters only.

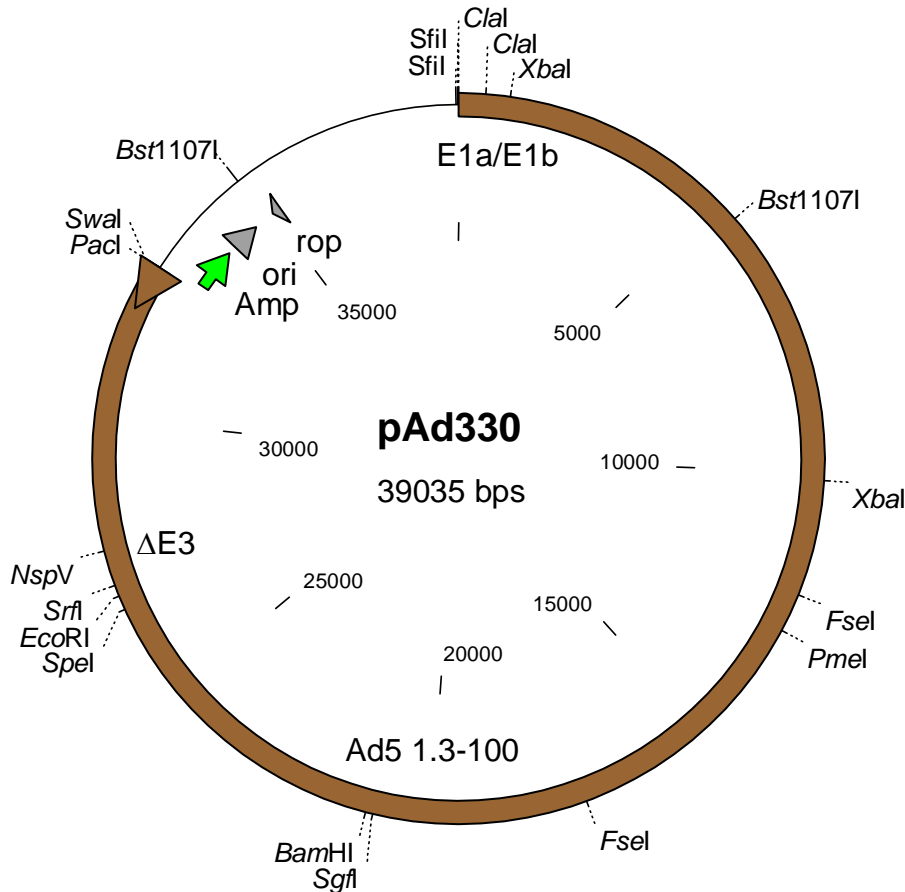
# pAd330

39,035 base pairs

Sequence available at [www.od260.com](http://www.od260.com)

pAd330 is a 39 kb plasmid that contains the sequences encompassing bp 466-right end (1.3-100 mu) of the Ad5 genome. The two *SfiI* sites naturally present in WT Ad5 DNA were mutated by substituting A for G and C at positions 16291 and 16294 in the Ad5 genome, and C and G for respectively G and C at positions 23001 and 23004 in the Ad5 genome, introducing silent mutations in the adenovirus pVII and DNA-binding protein coding sequences. A pair of *SfiI* sites that allow for directional cloning replaces the E1 promoter. The size of the E3 region deletion is 2.7 kb. The right ITR is flanked by *PacI* and *SwaI* sites. pAd330 is used in combination with the shuttle vector pE1.1 to construct conditionally replicative adenoviruses (CrAds) containing a heterologous promoter in front of the E1a TATA box. The maximum capacity for insertion is 4.8 kb.

| Feature               | Coordinates   | Source     |
|-----------------------|---------------|------------|
| 1.3-100 mu            | 13-32,811     | Ad5        |
| Right ITR             | 32,710-32,811 | Ad5        |
| origin of replication | 33,993-34,581 | pUC19      |
| Amp <sup>r</sup>      | 32,962-33,822 | <i>Tn3</i> |



Single and double cutters only.

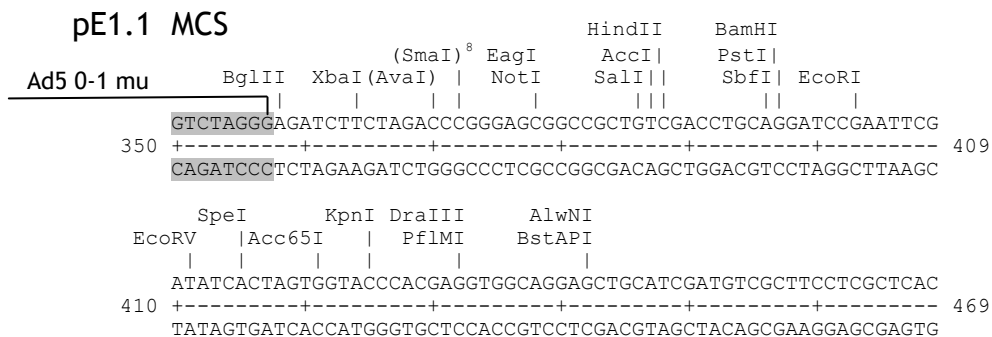
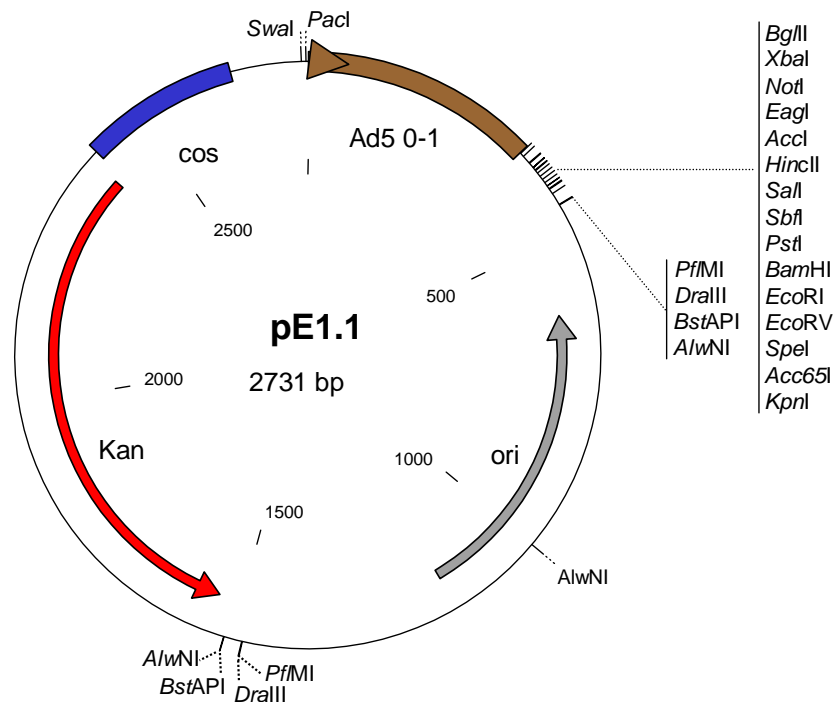
# pE1.1

2,731 base pairs

Sequence available at [www.od260.com](http://www.od260.com)

pE1.1 is a shuttle vector designed for inserting expression cassettes in place of the E1 region of the Ad5 genome, in combination with the AdenoQuick1.1 and AdenoQuick1.2 plasmids. It contains the first 353 base pairs (map unit 0-1) from the Ad5 genome (including the left ITR and packaging signal), preceded by *PacI* and *SwaI* sites, and followed by a multiple cloning site. Expression cassettes inserted into this site should contain a promoter, coding sequence and a polyA signal. The sequences encompassing the kanamycin-resistance gene, the  $\lambda$  cos site, the adenovirus 0-1 map units and the multiple cloning site are flanked by two sets of restriction sites *AlwNI*, *BstAPI*, *DraIII* and *PfI*MI which generate incompatible and non-symmetrical sticky ends.

| Feature               | Coordinates | Source          |
|-----------------------|-------------|-----------------|
| left ITR              | 5-107       | Ad5             |
| 0-1 mu                | 5-357       | Ad5             |
| origin of replication | 1220-632    | pUC19           |
| Kan <sup>r</sup>      | 2349-1534   | <i>Tn903</i>    |
| Cos                   | 2412-2659   | Phage $\lambda$ |



<sup>8</sup> Restriction sites between brackets are not unique.





## G. Product Descriptions and Other Useful Information

### AdenoQuick1.1 Kit      Cat # QK-01

The AdenoQuick1.1 Kit contains the components necessary to construct Ad5-based adenovirus vectors containing transgenes in place of the E1 region and characterized by a wild-type E3 region. The cloning strategy is based on the directional ligation of restriction fragments obtained using restriction enzymes that generate non-palindromic sticky ends. The cloning capacity is 5.2 kb. The kit contains enough materials to construct 10 recombinant viruses.

In addition to the shuttle vector pE1.1 and the adenovirus plasmid AdenoQuick1.1, a plasmid-based adenovirus DNA (Ad $\beta$ gal) is provided. It can be used as a control to monitor the transfection efficiency and will generate an adenovirus expressing a  $\beta$ -galactosidase under the control of a CMV promoter.

| Contents:                    | Cat. # | Quantity                                      |
|------------------------------|--------|---|
| • pE1.1                      | QP-01  | 20 $\mu$ g                                    |
| • AdenoQuick1.1 (WT E3)      | QR-01  | 2.5 $\mu$ g (good for 10 virus constructions) |
| • Control Ad $\beta$ gal DNA | ZC-01  | 10 $\mu$ g (good for 5 transfections)         |
| • Manual                     | QM-01  |   |

[Price List](#)

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[How to order](#)

### AdenoQuick1.2 Kit      Cat # QK-02

The AdenoQuick1.2 Kit contains the components necessary to construct Ad5-based adenovirus vectors containing transgenes in place of the E1 region and characterized by a 2.7 kb deletion in the E3 region. The cloning strategy is based on the directional ligation of restriction fragments obtained using restriction enzymes that generate non-palindromic sticky ends. The cloning capacity is 7.9 kb. The kit contains enough materials to construct 10 recombinant viruses.

In addition to the shuttle vector pE1.1 and the adenovirus plasmid AdenoQuick1.2, a plasmid-based adenovirus DNA (Ad $\beta$ gal) is provided. It can be used as a control to monitor the transfection efficiency and will generate an adenovirus expressing a  $\beta$ -galactosidase under the control of a CMV promoter.

| Contents:                      | Cat. # | Quantity                                |
|--------------------------------|--------|---|
| • pE1.1                        | QP-01  | 20 $\mu$ g                              |
| • AdenoQuick1.2 ( $\Delta$ E3) | QR-02  | 2.5 $\mu$ g (good for 10 constructions) |
| • Control Ad $\beta$ gal DNA   | ZC-01  | 10 $\mu$ g (good for 5 transfections)   |
| • Manual                       | QM-01  |   |

[Price List](#)

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[How to order](#)

**AdenoQuick1-Twin Kit****Cat # QK-03**

The AdenoQuick1-Twin Kit contains the components necessary to construct Ad5-based adenovirus vectors containing transgenes in place of the E1 region and characterized by either an intact or a deleted E3 region. The cloning strategy is based on the directional ligation of restriction fragments obtained using restriction enzymes that generate non-palindromic sticky ends. The cloning capacity is up to 7.9 kb. The kit contains enough materials to construct 20 recombinant viruses.

In addition to the shuttle vector pE1.1 and the adenovirus plasmids AdenoQuick1.1 and AdenoQuick1.2, a plasmid-based adenovirus DNA (Ad $\beta$ gal) is provided. It can be used as a control to monitor the transfection efficiency and will generate an adenovirus expressing a  $\beta$ -galactosidase under the control of a CMV promoter.

| <b>Contents:</b>               | <b>Cat. #</b> |   |
|--------------------------------|---------------|---|
| • pE1.1                        | QP-01         | 40 $\mu$ g                              |
| • AdenoQuick1.1 (WT E3)        | QR-01         | 2.5 $\mu$ g (good for 10 constructions) |
| • AdenoQuick1.2 ( $\Delta$ E3) | QR-02         | 2.5 $\mu$ g (good for 10 constructions) |
| • Control Ad $\beta$ gal DNA   | ZC-01         | 10 $\mu$ g (good for 5 transfections)   |
| • Manual                       | QM-01         |   |

[Price List](#)

-

[How to order](#)**AdenoQuick13.1 Kit****Cat # QK-04**

The AdenoQuick13.1 Kit contains the components necessary to construct Ad5-based bipartite adenovirus vectors containing transgenes in place of the E1 and E3 regions. The cloning strategy is based on the directional ligation of restriction fragments obtained using restriction enzymes that generate non-palindromic sticky ends. The cloning capacity is 7.7 kb. The kit contains enough materials to construct 10 recombinant viruses.

In addition to the shuttle vectors pE1.2 and pE3.1, and the adenovirus plasmid AdenoQuick13.1, a plasmid-based adenovirus DNA (Ad $\beta$ gal) is provided. It can be used as a control to monitor the transfection efficiency and will generate an adenovirus expressing a  $\beta$ -galactosidase under the control of a CMV promoter.

| <b>Contents:</b>                | <b>Cat. #</b> |   |
|---------------------------------|---------------|---|
| • pE1.2                         | QP-02         | 20 $\mu$ g                                    |
| • pE3.1                         | QP-03         | 20 $\mu$ g                                    |
| • AdenoQuick13.1 ( $\Delta$ E3) | QR-03         | 2.5 $\mu$ g (good for 10 virus constructions) |
| • Control Ad $\beta$ gal DNA    | ZC-01         | 10 $\mu$ g (good for 5 transfections)         |
| • Manual                        | QM-01         |   |

[Price List](#)

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[How to order](#)

**AdenoQuick1.1 DNA****Cat # QR-01**

AdenoQuick1.1 is a 39,332 bp plasmid that contains the sequences encompassing bp 3504-right end of the Ad5 genome. Two *SfiI* sites that allow for directional cloning replace the E1 region. The E3 region is intact. The right ITR is flanked by *PacI* and *SwaI* sites. AdenoQuick1.1 is used in combination with the shuttle vectors pE1.1, pE1.11 or pE1.12 to construct replication-deficient adenoviruses containing transgenes in place of the E1 region. The maximum transgene capacity is 5.2 kb. The vector is provided pre-digested with *SfiI*.

**Contents:**

- |                         |               |  |
|-------------------------|---------------|--|
| <b>Cat. #</b>           | <b>Cat. #</b> | <b>Cat. #</b>                            |
| • AdenoQuick1.1 (WT E3) | QR-01         | 2.5 µg (good for 10 virus constructions) |

[Price List](#)

-

[How to order](#)**AdenoQuick1.2 DNA****Cat # QR-02**

AdenoQuick1.2 is a 36,661 bp plasmid that contains the sequences encompassing bp 3504-right end of the Ad5 genome. Two *SfiI* sites that allow for directional cloning replace the E1 region. The E3 region is deleted (2.7 kb). The right ITR is flanked by *PacI* and *SwaI* sites. AdenoQuick1.2 is used in combination with the shuttle vectors pE1.1, pE1.11 or pE1.12 to construct replication-deficient adenoviruses containing transgenes in place of the E1 region. The maximum transgene capacity is 7.9 kb. The vector is provided pre-digested with *SfiI*.

**Contents:**

- |                       |               |  |
|-----------------------|---------------|--|
| <b>Cat. #</b>         | <b>Cat. #</b> | <b>Cat. #</b>                            |
| • AdenoQuick1.2 (ΔE3) | QR-02         | 2.5 µg (good for 10 virus constructions) |

[Price List](#)

-

[How to order](#)**AdenoQuick13.1 DNA****Cat # QR-03**

AdenoQuick13.1 is a 36,713 bp plasmid that contains the sequences encompassing bp 3504-right end of the Ad5 genome, with a 2.7 kb E3 deletion. Two pairs of *SfiI* sites that allow for directional cloning replace the E1 and E3 regions. The right ITR is flanked by *PacI* and *SwaI* sites. AdenoQuick13.1 is used in combination with the shuttle vectors pE1.2 and pE3.1 to construct replication-deficient adenoviruses containing transgenes in both the E1 and E3 regions. The maximum transgene capacity is 7.7 kb.

**Contents:**

- |                          |               |  |
|--------------------------|---------------|--|
| <b>Cat. #</b>            | <b>Cat. #</b> | <b>Cat. #</b>                            |
| • AdenoQuick13.1 (WT E3) | QR-03         | 2.5 µg (good for 10 virus constructions) |

[Price List](#)

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[How to order](#)

## pAd328 DNA      Cat # OP-01

pAd328 is a 39 kb plasmid that contains the sequences encompassing bp 466-right end (1.3-100 mu) of the Ad5 genome. The two *Sfi*I sites naturally present in WT Ad5 DNA were mutated by substituting A for G and C at positions 16291 and 16294 in the Ad5 genome, and C and G for respectively G and C at positions 23001 and 23004 in the Ad5 genome, introducing silent mutations in the adenovirus pVII and DNA-binding protein coding sequences. Two pairs of *Sfi*I sites that allow for directional cloning replace the E1 promoter and the E3 region. The size of the E3 region deletion is 2.7 kb. The right ITR is flanked by *Pac*I and *Swa*I sites. pAd328 is used in combination with the shuttle vectors pE1.2 and pE3.1 to construct conditionally replicative adenoviruses (CrAds) containing a heterologous promoter in front of the E1a TATA box, and a transgene in place of the E3 region. The maximum combined promoter/transgene capacity is 4.6 kb. pAd328 is provided digested with *Sfi*I.

|                  |               |  |
|------------------|---------------|--|
| <b>Contents:</b> | <b>Cat. #</b> |  |
| • pAd328 (Δ E3)  | OP-01         | 5 μg (good for 10 virus constructions) |

[Price List](#)

-

[How to order](#)

## pAd329 DNA      Cat # OP-02

pAd329 is a 41.7 kb plasmid that contains the sequences encompassing bp 466-right end (1.3-100 mu) of the Ad5 genome. The two *Sfi*I sites naturally present in WT Ad5 DNA were mutated by substituting A for G and C at positions 16291 and 16294 in the Ad5 genome, and C and G for respectively G and C at positions 23001 and 23004 in the Ad5 genome, introducing silent mutations in the adenovirus pVII and DNA-binding protein coding sequences. A pair of *Sfi*I sites that allow for directional cloning replaces the E1 promoter. The E3 region is intact. The right ITR is flanked by *Pac*I and *Swa*I sites. pAd329 is used in combination with the shuttle vector pE1.1 to construct conditionally replicative adenoviruses (CrAds) containing a heterologous promoter in front of the E1a TATA box. The maximum capacity for insertion is 2.1 kb.

|                  |               |  |
|------------------|---------------|--|
| <b>Contents:</b> | <b>Cat. #</b> |  |
| • pAd329 (WT E3) | OP-02         | 5 μg (good for 10 virus constructions) |

[Price List](#)

-

[How to order](#)

## pAd330 DNA      Cat # OP-03

pAd330 is a 39 kb plasmid that contains the sequences encompassing bp 466-right end (1.3-100 mu) of the Ad5 genome. The two *Sfi*I sites naturally present in WT Ad5 DNA were mutated by substituting A for G and C at positions 16291 and 16294 in the Ad5 genome, and C and G for respectively G and C at positions 23001 and 23004 in the Ad5 genome, introducing silent mutations in the adenovirus pVII and DNA-binding protein coding sequences. A pair of *Sfi*I sites that allow for directional cloning replaces the E1 promoter. The size of the E3 region deletion is 2.7 kb. The right ITR is flanked by *Pac*I and *Swa*I sites. pAd330 is used in combination with the shuttle vector pE1.1 to construct conditionally replicative adenoviruses (CrAds) containing a heterologous promoter in front of the E1a TATA box. The maximum capacity for insertion is 4.8 kb.

|                         |               |   |
|-------------------------|---------------|---|
| <b>Contents:</b>        | <b>Cat. #</b> |   |
| • pAd330 ( $\Delta$ E3) | OP-03         | 5 $\mu$ g (good for 10 virus constructions) |

[Price List](#) - [How to order](#)

### **pE1.1      Cat # QP-01**

---

pE1.1 (2731 bp) is a shuttle vector designed for inserting expression cassettes into the E1 region of AdenoQuick1.1 and AdenoQuick1.2. It contains the first 353 nucleotides from the Ad5 genome, preceded by *PacI* and *SwaI* sites, and followed by a multiple cloning site. The sequences encompassing the kanamycin-resistance gene, the  $\lambda$  cos site, the adenovirus 0-1 map units and the multiple cloning site are flanked by two sets of restriction sites *A**l**u**N**I*, *B**s**t**A**P**I*, *D**r**a**l**l**I* and *P**f**I**M**I*.

|                  |               |            |
|------------------|---------------|------------|
| <b>Contents:</b> | <b>Cat. #</b> |            |
| • pE1.1          | QP-01         | 20 $\mu$ g |

[Price List](#) - [How to order](#)

### **pE1.2      Cat # QP-02**

---

pE1.2 (2432 bp) is a shuttle vector designed for inserting expression cassettes into the E1 region of AdenoQuick13.1. It contains the first 353 nucleotides from the Ad5 genome, preceded by *PacI* and *SwaI* sites, and followed by a multiple cloning site. The sequences encompassing the kanamycin-resistance gene, the adenovirus 0-1 map units and the multiple cloning site are flanked by two sets of restriction sites *A**l**u**N**I*, *B**s**t**A**P**I*, *D**r**a**l**l**I* and *P**f**I**M**I*.

|                  |               |            |
|------------------|---------------|------------|
| <b>Contents:</b> | <b>Cat. #</b> |            |
| • pE1.2          | QP-02         | 20 $\mu$ g |

[Price List](#) - [How to order](#)

### **pE3.1      Cat # QP-03**

---

pE3.1 (2308 bp) is a shuttle vector designed for inserting expression cassettes into the E3 region of AdenoQuick13.1. It contains a multiple cloning site flanked by a 180-bp  $\lambda$  cos site and a kanamycin-resistance gene. The sequences encompassing the cos site and the multiple cloning site are flanked by two sets of restriction sites *A**l**u**N**I*, *B**s**t**A**P**I*, *D**r**a**l**l**I* and *P**f**I**M**I*.

|                  |               |            |
|------------------|---------------|------------|
| <b>Contents:</b> | <b>Cat. #</b> |            |
| • pE3.1          | QP-02         | 20 $\mu$ g |

[Price List](#) - [How to order](#)

**Control Ad $\beta$ gal DNA****Cat # ZC-01**

---

The Control Ad $\beta$ gal DNA is a plasmid-isolated adenovirus DNA that contains a CMV-lacZ expression cassette in place of the E1 region. It can be used as a control to monitor the efficiency of transfection and virus recovery. It will generate a  $\beta$ galactosidase-expressing adenovirus useful for future experiments. Enough DNA is provided for 5 transfections.

**Contents:**

|                              | <b>Cat. #</b> |            |
|------------------------------|---------------|------------|
| • Control Ad $\beta$ gal DNA | ZC-01         | 10 $\mu$ g |

[Price List](#)

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[How to order](#)

## Price List<sup>11</sup>

| Product              | Cat # | Unit Price |
|----------------------|-------|------------|
| AdenoQuick1.1 Kit    | QK-01 | \$ 550.00  |
| AdenoQuick1.2 Kit    | QK-02 | \$ 550.00  |
| AdenoQuick1-Twin Kit | QK-03 | \$ 850.00  |
| AdenoQuick13.1 Kit   | QK-04 | \$ 550.00  |
| AdenoQuick1.1 DNA    | QR-01 | \$ 400.00  |
| AdenoQuick 1.2 DNA   | QR-02 | \$ 400.00  |
| pAd328 DNA           | OP-01 | \$ 400.00  |
| pAd329 DNA           | OP-02 | \$ 400.00  |
| pAd329 DNA           | OP-03 | \$ 400.00  |
| AdenoQuick13.1 DNA   | QR-03 | \$ 400.00  |
| pE1.1                | QP-01 | \$ 200.00  |
| pE2.1                | QP-02 | \$ 200.00  |
| pE3.1                | QP-03 | \$ 200.00  |
| Adβgal DNA           | ZC-01 | \$ 200.00  |

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### Fax Ordering (24 hour)

1-208-345-7569

Please use the attached Fax order form.

### Email Ordering

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<sup>11</sup> As of January 1, 2008. Prices are subject to change without prior notice.

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