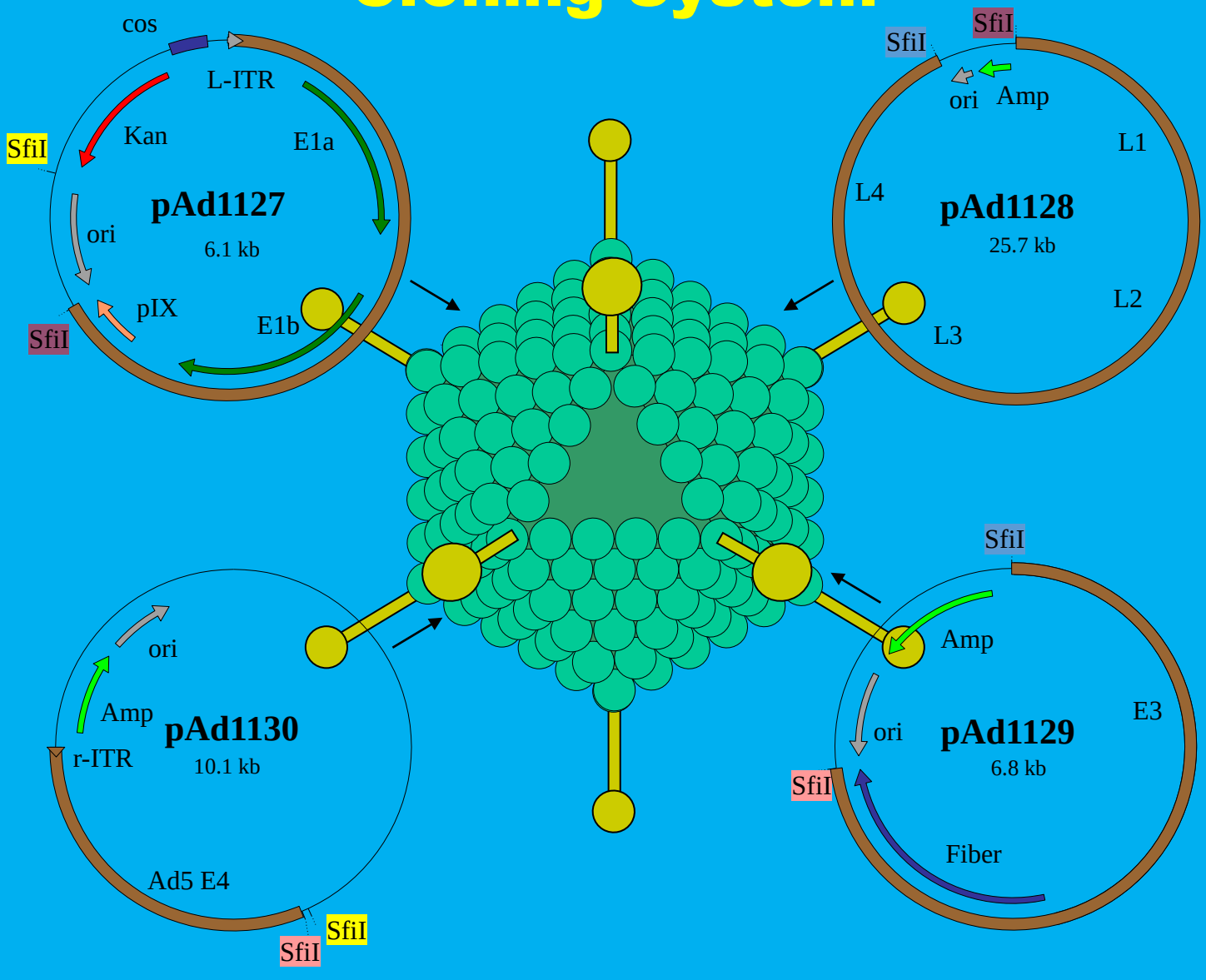
OD260 Inc.

The AdenoQuick2.0 Cloning System



Important Notice

Before opening the kit:

- Read our License Agreement (p. 3)
- Read the entire manual, especially the chapter “Biosafety Information” (p. 13).
- Become familiar with the different techniques used, especially those related to the manipulation of adenovirus (p. 14).
- 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://osp.od.nih.gov/office-biotechnology-activities/biosafety/nih-guidelines)

License Agreement

The purchased kit consists of the Materials identified in Section 2 (p. 12 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:

- 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.
- 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.
- The Recipient shall hold OD260 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.
- In view of the Materials' experimental nature, OD260 Inc. provides no warranty, express or implied, including any warranty of merchantability or fitness for a particular purpose or warranty against infringement.
- All unused supplies of the Materials and Derivatives will, at OD260 Inc.'s option, be destroyed or returned to OD260 Inc., when the investigation for which they have been purchased discontinues or is terminated.
- 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 sage use of biologicals, including Adenovirus-based biologicals.
- This agreement is between the Recipient and OD260 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 OD260 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 OD260 Inc.

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1 INTRODUCTION

1.1 DESCRIPTION

AdenoQuick2.0 is a modular method for constructing recombinant adenovirus vectors. It uses a set of 4 plasmids that contain different regions of the virus genome. These regions can be manipulated independently, then assembled together by directional ligation to reconstitute in a cosmid the entire genome of the desired virus. The cosmid DNA is linearized and transfected into helper cells in order to rescue the virus.

There are 4 basic plasmids: pAd1127, pAd1128, pAd1129, and pAd1130. pAd1127 contains the Ad5 left ITR, packaging signal, the E1 region and the pIX gene. pAd1128 encompasses the E2 region and most of the late genes. pAd1129 includes the E3 region and fiber gene. pAd1130 contains the E4 region and the right ITR. The adenovirus sequences are excised from these 4 plasmids (or their derivatives) using the restriction enzyme SfiI, and are ligated together to reconstitute the entire genome of the recombinant adenovirus. The ligation product is packaged into phage Lambda, which is subsequently used to infect *E. coli*, generating a cosmid. The entire process is extremely efficient because each of the SfiI sites present in the 4 plasmids generates a unique non-palindromic sticky end that enables directional ligation between four DNA fragments. Also, the method selects clones containing full-size genomes because phage λ packages DNA's ranging from 39 to 54 kb.

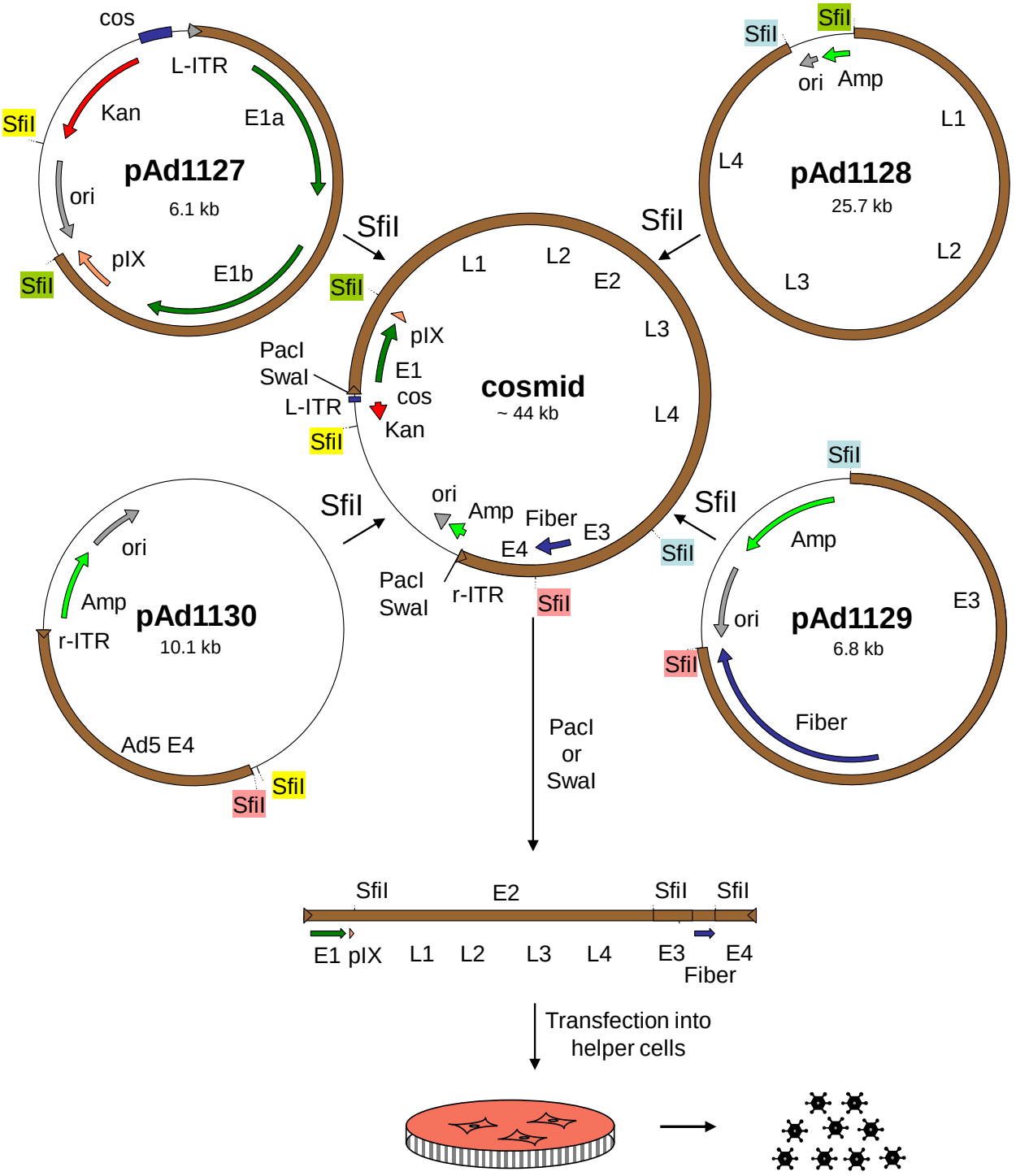


Figure 1: Overview of the AdenoQuick2.0 cloning system. The first step consists in modifying one or several of 4 shuttle plasmids (or derivatives) according to your needs: pAd1127 for the left ITR, packaging signal, E1 region, and pIX; pAd1128 for the E2 region and most late genes; pAd1129 for the E3 region and fiber; pAd1130 for E4 region and the right ITR. The 4 shuttle plasmids are then digested with SfiI. The DNA fragments of interest are gel-purified and ligated with each other in order to reconstitute in a cosmid the entire sequence of your recombinant adenovirus. The cosmid is linearized with PacI or SwaI, and transfected into helper cells in order to rescue the virus.

1.2 APPLICATIONS

The applications of AdenoQuick2.0 are numerous:

- **E1-substituted vectors**, also known as "first-generation" adenovirus vectors, in which an expression cassette is inserted in place of the E1a/b genes. The position and the size of the E1 deletion can vary. Vectors with smaller E1 deletions have usually better yields. Vectors with larger E1 deletions are less prone to generate replication-competent viruses (RCA). **RCA-free vectors** can be obtained if they are amplified in compatible cell lines such as Per.C6®.

- **E3-substituted vectors**, in which expression cassettes are inserted in place of the E3 region. This type of insertion can be useful to construct bipartite viruses or armed oncolytic vectors (see below). It can also be used to avoid the emergence of replication-competent adenoviruses (RCA) during virus amplification in 293 cells. For doing so, the expression cassette of interest is inserted into the E3 region, while the E1 region is kept empty. If necessary the size of the vector is brought to about 36 kb using stuffer DNA. If the viral vector recombines with the WT E1 region inserted into the 293 cell chromosome, a viral genome will be generated, which will be too long to be packaged, and will not generate viral particles. Please note that there are some restrictions about the nature of the expression cassette that can be inserted into the E3 region. Two E3 deletions are available, with sizes of 1.9 and 2.7 kb.

- **E4-deleted vectors**, also with various deletions (1.2 and 2.8 kb). These vectors are usually used in combination with large deletions in the E1 and E3 regions to accommodate long expression cassettes. Vectors with the 1.2 kb E4 deletion can grow in standard 293 cells, while those with the 2.8 kb deletion necessitate a cell line that expresses the E4 genes *in trans*. E1/E3/E4-deleted vectors have a maximum of **10.4 kb cargo** capacity.

- **Bipartite viruses**, containing two expression cassettes, one in the E1 region, and the other in the E3 region. Applications include:

    - Vectors containing your gene of interest in the E1 region, and a reporter gene in the E3 region, or vice-versa

    - Vectors containing inducible expression systems

    - Vectors expressing a regulator and a regulated gene

- Vectors expressing two genes involved in the same pathway
- Vectors expressing two antigens

Bipartite viruses have several advantages over using two vectors each expressing a single transgene. They are less expensive to produce, since only one round of virus amplification/purification/titration is necessary. Also, they should be less immunogenic when injected in vivo, since less virus particles need to be injected.

- **Oncolytic**, conditionally-replicative vectors (**CrAds**), containing specific promoters controlling the expression of the E1 and/or E4 regions, and with the possibility of inserting additional genes in the E3 region **("armed" CrAds**).
- Fiber, pIX, hexon mutants for **vector targeting.** The natural tropism of wild-type Ad5 is mediated by the binding of fiber knob to the cocksakie-adenovirus receptor (CAR). That tropism can be re-directed to specific cell types or tissues by ligands in the fiber, pIX, or hexon genes.
- **Helper viruses** used for the production of helper-dependent (gutless) adenovirus vectors. Their packaging signal can be easily modified to favor the packaging of the gutless vector over that of the helper virus.
- Any viable combination of the above, or any mutation in a region of the genome yet to be explored.

1.3 ADVANTAGES

Using AdenoQuick2.0 to construct adenovirus vectors has many advantages:

- It is a **modular method** that enables assembling variants of a virus by swapping blocks. OD260 Inc has constructed and tested numerous derivatives of pAd1127, pAd1128, pad1129, and pAd1130. The small size of these vectors facilitates the introduction of mutations in any place of the viral genome. For instance, it allows for engineering a virus targeted to a specific tissue using an E1-deleted backbone, then convert it to helper virus for gutless virus production. Or it allows combining features from several viruses into one backbone.

- It is a **very easy and efficient construction method**, based on the directional ligation of multiple DNA fragments, and relying upon the fact that only full-size genome will be packaged into lambda phage particles.
    - Cosmid technology is particularly well suited for the cloning of the 36 kb-long adenoviral genome in *E. Coli*. Because phage λ packages DNA's ranging from 39 to 54 kb, the method selects clones containing full-size genomes.
    - No homologous recombination event is necessary. Therefore is no danger of an unpredicted recombination in *E. coli* that would be not detected unless by sequencing and would render the DNA non-infectious. There is no need either for the transformation of a *recA*$^+$ strain (eg: BJ5183) for the recombination and the subsequent transfer to a *recA endA* strain for plasmid preparation. Phage λ infection can be performed directly into *recA endA* strains such as DH5α, XL-1 blue or Top10.

- Compared to other techniques, this method requires **less "hands-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 minimum number of E. coli clones needs to be analyzed.
    - Yield is high (up to 3 μg cosmid DNA/mL bacterial culture). Therefore a small-scale culture will provide enough DNA for rescuing the virus.

2 PRODUCT COMPONENTS

AdenoQuick2.0 **size:10 reactions** **cat # AQ-2**

The AdenoQuick2.0 kit contains the reagents necessary for constructing at least 10 recombinant adenoviruses. The kit can be customized by choosing 4 shuttle plasmids from our collection, one from each of the pAd1127, pAd1128, pAd1129, and pAd1130 categories.

Product	Use	Size	Cat #
pAd1127 or derivative	Shuttle plasmid E1 region + pIX	20 µg	www.od260.com
pAd1128 or derivative	Shuttle plasmid E2 region + late genes	20 µg	www.od260.com
pAd1129 or derivative	Shuttle plasmid E3 region + Fiber	20 µg	www.od260.com
pAd1130 or derivative	Shuttle plasmid E4 region	20 µg	www.od260.com
Lambda packaging reagents, including:	Cosmid construction	10 reactions	R-1001
• Packaging extract	Cosmid construction	50 µL	
• 1M MgSO4	Cosmid construction	500 µL	
• 10% Maltose	Cosmid construction	500 µL	
• SM medium	Cosmid construction	1.3 mL	
Ad-βGal DNA	Control for virus rescue by transfection	10 µg	ZC-01
Handbook		1	

Each item can be ordered individually.

Storage conditions: -70ºC

3 BIOSAFETY INFORMATION

3.1 EPIDEMIOLOGY AND CLINICAL MANIFESTATIONS OF ADENOVIRUS

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.

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 cystids cause 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.

3.2 REQUIRED FACILITIES AND EQUIPMENT

The National Institute of Health has designated adenovirus as Level 2 biological agent. 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:

- 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

3.3 PRECAUTIONS TO TAKE WHILE WORKING WITH ADENOVIRUS

Work with adenovirus must be performed in a BL2 lab. There you must:

- 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.

3.4 SPILL RESPONSE

- Treat liquid spills immediately with at least one volume of 10% bleach. If the spill is large, prevent it from spreading further with wipes. 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.

3.5 MORE READINGS…

For further information about biosafety, we recommend reading the following publications from the NIH Division of Safety (http://osp.od.nih.gov/office-biotechnology-activities/rdna.htm):

- Biosafety in Microbiological and Biomedical Laboratories (http://www.cdc.gov/biosafety/publications/bmbl5/)
- NIH guidelines for research involving recombinant DNA molecules (http://osp.od.nih.gov/office-biotechnology-activities/biosafety/nih-guidelines)

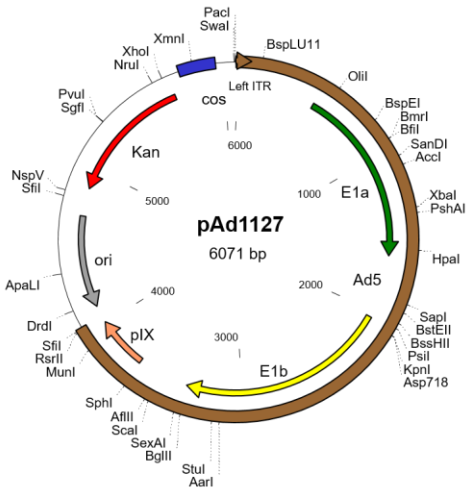
Consult also your regional and institutional guidelines.

4 PROTOCOLS

4.1 SHUTTLE PLASMID CONSTRUCTION

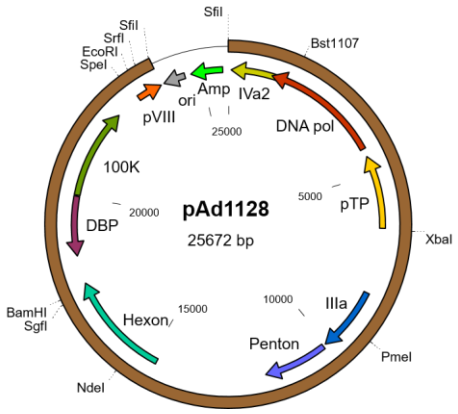
The first step towards the generation of your adenovirus vector is the construction of shuttle plasmids. There are 4 series of shuttle plasmids. You will need one plasmid from each set to construct your vector.

pAd1127 Series



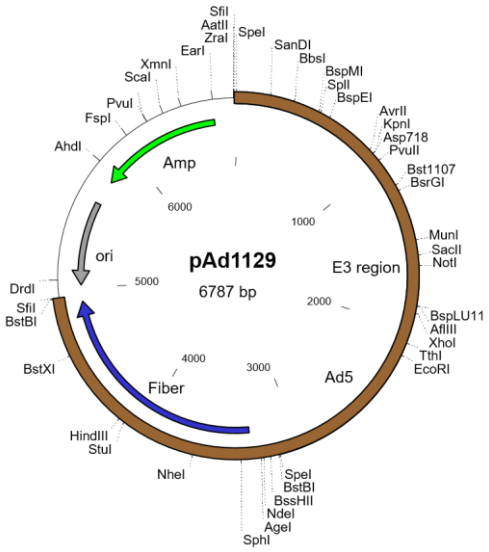
pAd1127 contains the left-end Ad5 sequence including the left ITR, packaging signal, the entire E1 region and the pIX gene. Many derivatives exist already, which differ by the packaging signal (repeats A1-A5 or A1-A7), the extent of the E1 deletion (starting at psn 350 or 450 in the Ad5 genome, and ending at psn 3300 or 3500), the presence of empty expression cassettes (CMV, RSV…) etc…

pAd1128 Series



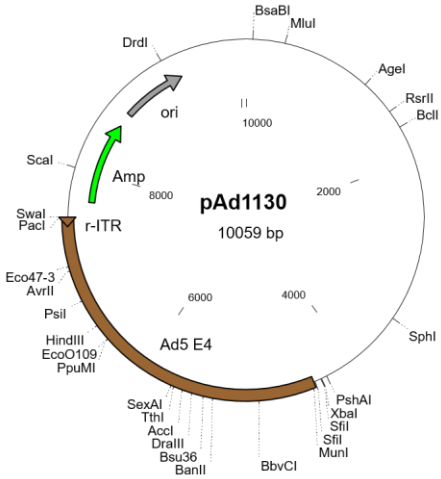
pAd1128 contains the Ad5 E2 region (DNA polymerase, terminal protein, DNA-binding protein) and most of the late genes.

pAd1129 Series



pAd1129 contains the entire Ad5 E3 region between SpeI sites and the fiber gene between BstBI sites. Many derivatives are already available: deletions in the E3 region (1.9 or 2.7 kb), expression cassettes (RSV or CMV promoters), hybrid fibers (e.g. Ad5/3, Ad5/35), etc…

pAd1130 Series



pAd1130 contains the Ad5 E4 region and the right ITR. Derivatives have large deletions (1.2 or 2.8 kb) or multiple cloning sites downstream from the ITR for inserting specific promoters to control the expression of the E4 genes.

The list of all variants of pAd1127, pAd1128, pAd1129, and pAd1130 is growing and can be found on our web site. Please use of our Vector Selection tool in order to find the plasmid that best fits your needs.

- Modify your shuttle plasmid(s) using standard recombinant DNA techniques. Verify its integrity by restriction analysis and/or sequencing.

  ☺ If you plan to construct a mono- or dicistronic adenovirus expression vector, make sure that your transgene has his own promoter and poly(A) signal. Do not rely on the adenovirus transcriptional elements for strong expression. The adenovirus left ITR and packaging signal contain enhancer elements of the E1a promoter. These elements are unable to drive strong expression of a downstream coding sequence, but are able to influence the activity of adjacent promoters. If you are using a regulated promoter (e.g. tissue-specific), you might want to orient your expression cassette towards the left end of the adenovirus genome in order to minimize the influence of these E1a enhancers on the activity of your promoter.

- Digest the resulting plasmid with SfiI and purify the DNA fragment containing the adenovirus sequences on agarose gel.

  ☺ SfiI (NEB # R0123S) is an enzyme that recognizes an 8 bp-long interrupted palindrome. Upon cutting, it creates a 3 nt-long sticky end with a 3' extension, the sequence of which can be chosen arbitrarily. The sequences of the SfiI sites contained in plasmids pAd1127, pAd1128, pAd1129 and pAd1130 have been designed so that the sticky end generated at the end of the pIX gene in pAd1127 will anneal only with the sticky end generated at the start of the Ad5 sequence in pAd1128, and so on. This 4-fragment directional ligation is very efficient in assembling the entire genome of your recombinant adenovirus in a cosmid.

  ☺ If the heterologous sequence that you are inserting in a shuttle plasmid contains an SfiI site, the digestion of the resulting plasmid with SfiI will generate 3 fragments. You will have to purify the two fragments of interest on agarose gel and ligate them with the SfiI fragments purified from the other three shuttle plasmids. The ligation reaction will thus contain 5 fragments. If all sticky ends are different, the cloning efficiency will remain very high. If the fifth sticky end can anneal with one of the others, the cloning efficiency will decrease. You might have to analyze more clones, but you will still be able to obtain the correct cosmid.

4.2 COSMID 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.

This is performed in three steps:

1. Digesting the pAd1127, pAd1128, pAd1129, and pAd1130 plasmid derivatives with SfiI, purifying the fragments of interest on agarose gel and ligating them to each other.
2. Packaging the ligation products into phage λ
3. Infecting E. coli with the λ packaging products.

This method is very efficient, even if it involves a 4-fragment ligation. The ligation of the SfiI fragments is directional. And since phage λ packages preferentially DNA molecules that are 39-54 kb long, it selects for clones that carry full-size genomes. Almost 100% of the bacterial clones will carry the correct cosmid.

4.2.1 DAY 1

□ Digest 5 µg each plasmid from the pAd1127, pAd1128, pAd1129, and pAd1130 series with SfiI.

☺ A good source of SfiI is New England Biolabs (Cat # R0123). The enzyme works most efficiently at 50 ºC. An efficient digestion will be performed in an oven instead of a water bath. In a water bath, H_2O from the reaction will evaporate and condense on the cap of the tube, thereby increasing the salt concentration of the reaction and inhibiting the enzyme.

☺ Example:
- 5 µL plasmid DNA (1 µg/µL)
- 38 µL H_2O
- 5 µL (10x) NEB2 buffer
- 2 µL SfiI (20 units/µL)
- Incubate for 1 to 2 hours at 50ºC in an oven

□ Separate the digestion products on a preparative 0.7% agarose gel in TAE buffer.

□ Excise the fragments of interest, and extract the DNA from agarose using a gel extraction kit (for instance QIAquick: QIAgen Cat # 28704). Elute the DNA from the mini-columns in 30 µL elution buffer.

☺ Kits that use silica spin columns such as QIAquick works well with the 20 kbp SfiI fragment from pAd1128, even though the manufacturer rates its efficiency between 100 bp and 10 kb.

☺ Check the quality and the fluorescence intensity of the purified DNA fragments on agarose gel. It is important to perform the ligation with intact fragments, and to use large amounts of DNA. Working with too small DNA amounts will lower the cloning efficiency.

- □ Ligate 2 µL *Sfi*I fragments from the pAd1127, pAd1128, pAd1129, and pAd1130 derivatives in a 10 µL volume, and incubate overnight at 16ºC.

  ☺ Example of ligation reaction:
  - 2 µL pAd1127 SfiI fragment
  - 2 µL pAd1128 SfiI fragment
  - 2 µL pAd1129 SfiI fragment
  - 2 µL pAd1130 SfiI fragment
  - 1 µL T4 ligase buffer
  - 1 µL T4 ligase (400 units/µL, NEB cat #M0202)
  - Incubate overnight at 16 ºC

  ☺ The ligation can also be performed for 1 hour at room temperature.

- □ Streak *E. coli* from a glycerol stock on a LB plate. Incubate overnight at 37 °C.

  ☺ We recommend XL1-blue (Stratagene/Agilent) and Top 10 (Invitrogen/Life Technologies): cosmids are stable in these strains and DNA yields are high. DH5α is somewhat less efficient.

4.2.2 DAY 2

First thing in the morning: Preparation of Competent Bacteria

- □ Prepare 2 mL LB supplemented with 0.2% maltose and 10 mM $MgSO_4$.

  ☺ Sterile 10% Maltose and 1 M MgSO4 solutions are provided in the AdenoQuick kits.
  ☺ Example:
  - 2 mL LB
  - 40 µL 10% maltose
  - 20 µL 1 M $MgSO_4$

- □ 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_{600}).

Packaging:

- □ Add 2 µL ligation mixture to a microcentrifuge tube.
- □ Thaw a vial of λ packaging extract (green vial), keeping it on ice as much as possible. Once thawed, add immediately 5 µL 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 one hour (not longer than 90 min).
- □ Add 100 µL SM buffer (provided in the kit) and 5 µL chloroform. Mix vigorously for a few seconds with your fingertips until white debris appear. Centrifuge for

30 sec., transfer the supernatant to a new tube (avoid the chloroform) and keep on ice or at 4 °C.

☺ Immediately after pipetting the λ packaging extract from the tube, quickly freeze the remainder in liquid N_2. The efficiency of the next reaction will not be significantly reduced.

☺ The packaged phage extract is stable for a few months at 4°C.

Infection:

□ Dilute the competent bacteria to OD_{600} = 1.0 in LB. Mix 50 µL packaging mixture with 50 µL of the diluted cells in a microcentrifuge tube and incubate in a 37°C water bath for 30 min. Avoid bubbles and do not spin down.

□ Spread the bacteria on a LB plate supplemented with 50 µg/mL ampicillin and 25 µg/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).

4.2.3 DAY 3

□ **First thing in the morning**: Inoculate 2 mL LB Lennox + 50 µg/mL ampicillin and 25 µg/mL kanamycin with several clones.

☺ The cloning efficiency of this system is very high (practically 100%). Therefore you need to analyze only 3-4 clones per construct.

☺ Use LB Lennox if you work with Top10.

□ **Afternoon**: Harvest bacteria when the cultures approach saturation.

□ Purify the cosmid DNAs and verify their identity by restriction analysis.

☺ Use your favorite method for plasmid purification. The standard alkaline lysis method works very well. We have used also 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.

- □ **Late evening**: Inoculate one of the correct clones (100 µL saturated culture) into 40 mL LB supplemented with 50 µg/mL ampicillin and 25 µg/mL kanamycin.

  ☺ We recommend that once you get the *E. coli* clones carrying your cosmid, 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.

4.2.4 DAY 4

- □ Harvest the cells at the end of the exponential growth phase, i.e. when OD_{600} is between 2.0 and 2.5 (usually after 8-12 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. Do not grow for more than 12 hours.

  ☺ A 40-mL culture should provide enough DNA for the virus recovery step.

- □ 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.

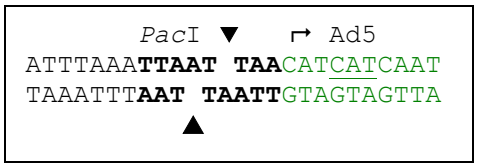
  ☺ It is important to have a DNA as pure as possible, since its quality will affect the transfection efficiency and virus recovery. Avoid genomic DNA.

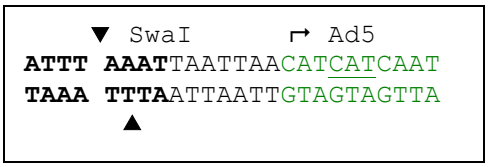
  ☺ Remember not to dry the cosmid too much after ethanol or isopropanol precipitation. Pipet cosmid solutions gently in order not to shear the DNA.

- □ Verify the integrity of your cosmid by restriction analysis.

- □ Digest 20 µg cosmid with either *Pac*I or *Swa*I, 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 *Pac*I and *Swa*I 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, *Pac*I and *Swa*I generate 3 and 11 nt-long hanging sequences, respectively:





The adenoviruses that will be generated from either *Pac*I or *Swa*I-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.[1]

The DNA ends generated by *Pac*I 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 *Pac*I- or *Swa*I-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.

4.3 VIRUS RECOVERY AND CHARACTERIZATION

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.

[1] King *et al*, EMBO J. 1994 Dec 1;13(23):5786-92.

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 2.

Briefly, two 293 cell dishes per construct are transfected with the *Pac*I- or *Swa*I-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 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.

* 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*.

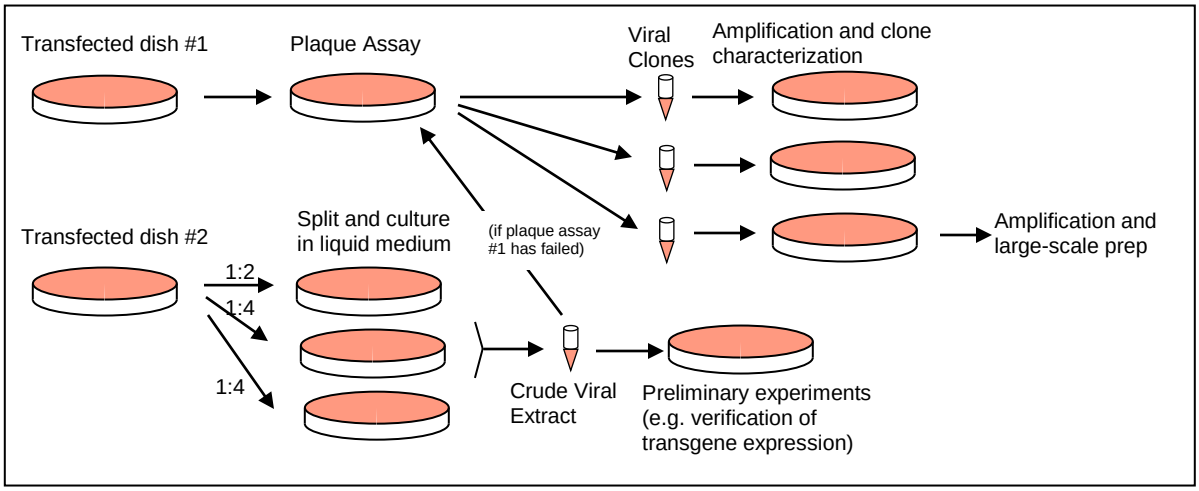


Figure 2: Proposed Flow-Chart to Isolate, Analyze and Amplify your Recombinant Virus.

4.3.1 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* β-galactosidase (AdβGal cat # ZC-01). This β-Gal virus should be useful in your future experiments.

4.3.2 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/μg DNA.

□ Prepare 2x HBS and $CaCl_2$ 2M:

2x HBS (25 mL): Add
- 1.4 mL NaCl 5 M
- 2.5 mL Hepes 0.5 M
- 75 µL Na_2HPO_4 (or NaH_2PO_4) 0.5 M
- H_2O to 22 mL

Adjust to pH 7.0
Adjust volume to 25 mL with H_2O
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:

Reagent	*Your Recombinant virus (Optional: in duplicate)*	*β-Gal control virus (Optional)*
H_2O	199 µL	215 µL
$CaCl_2$ 2M	31 µL	31 µL
DNA	20 µL	4 µL (2µg)
Total volume	**250 µL**	**250 µL**

□ In a 5-mL round-bottom polypropylene tube (e.g. Falcon, Becton-Dickinson # 35-2063), dispense 250 µL 2x HBS. Slowly add the $H_2O/CaCl_2$/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.

4.3.3 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 µL 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 µL virus extract supplemented with 250 µL DMEM/10% FBS (total = 500 µL). 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 or four days after the transfection, split the second 6-cm 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[2], 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

[2] Hirt B. (1967). J. Mol Biol 26:365-369.

appear, for up to 2 weeks. Feed the cells every 3-4 days with ¼ volume DMEM/10% FBS.

5 TROUBLESHOOTING

5.1 COSMID CONSTRUCTION

Observation	Possible Cause(s)	Comments & Suggestions
♦ **No colony recovered after >12 hours incubation at 37 ºC**	♦ Ligation was not efficient.	♦ Verify the identity, the quality and the amount of DNA fragments on agarose gel. ♦ Add equimolar amounts of DNA fragments in the ligation reaction. ♦ Use a new batch of 10 x T4 DNA ligase buffer, as the ATP it contains might be degraded. ♦ Use a new batch of T4 DNA ligase. ♦ Set up the ligation reaction at 16ºC overnight.
	♦ Packaging into phage λ was not efficient	♦ Use a new batch of λ packaging extract ♦ Avoid making bubbles when adding the ligation products to the packaging extract. Mix by pipeting in/out. Do not spin the packaging reaction. ♦ Incubate the packaging reaction at 30ºC for no more than 90 min. ♦ Use high-quality $CHCl_3$ to precipitate cell debris after packaging ♦ After pelleting the cell debris by a short centrifugation, transfer the packaged λ phage into a new tube and AVOID taking $CHCl_3$. ♦ Keep the packaged reaction on ice or at +4ºC until use.
	♦ Bacteria were not competent for phage λ infection	♦ Use a fresh colony of bacteria to inoculate 2 mL LB

		♦ Make sure to add 0.2% Maltose and 10 mM MgSO4 to the 2 mL LB. These reagents are provided in the kit.
	♦ *E. coli* antibiotic selection	♦ Use 50 µg/mL ampicillin and 25 µg/mL kanamycin ♦ Use LB Lennox instead of LB Miller
♦ **Too many colonies obtained after transformation (colonies are indistinguishable and form an almost continuous monolayer).**	♦ Antibiotic concentration too low, Petri dishes too old.	♦ Make new antibiotic solutions and new plates containing 50µg/mL ampicillin and 25 µg/mL kanamycin.
	♦ Contamination occurred with an amp^r and kan^r bacteria.	♦ Use bacteria from a reputable source.
♦ **The cosmid restriction pattern is not the one expected.**	♦ DNA recombination occurred during bacterial growth.	♦ Grow the bacteria for no more than 12 hours.
	♦ The enzyme you used for restriction analysis is methylation-dependent.	♦ Verify the sequence of your insert for the presence of the methylation site. Use another enzyme.
	♦ The plasmid you want to construct is unstable in E. coli, either because of DNA topology concerns or because an open reading frame is transcribed and translated into a toxic product.	♦ Insert your transgene in opposite orientation. ♦ Use an E. coli strain that has a reduced potential for homologous recombination (e.g.SURE cells – Agilent)

5.2 VIRUS RECOVERY

Observation	Possible Cause(s)	Comments & Suggestions
♦ **The entire cell monolayers are showing cytopathic effect 2-3 days after transfection.**	♦ The reagents used for transfection are toxic to the cells.	♦ Wash the cell monolayers twice with warm DMEM/FBS. If you have used the CaPO4-DNA co-precipitation method, this should have been done 8-12 hours after the transfection.

♦ **Do not get virus at all. No cytopathic effect is observed up to 2 weeks after the transfection.**	♦ One of your shuttle plasmids is not correct.	♦ Verify the identity of the shuttle vectors that you modified (restriction analysis, sequencing…)
	♦ The cosmid is not correct.	♦ Verify the identity of the cosmid that you constructed (restriction analysis, sequencing…). Compare the restriction patterns of several clones next to each other.
	♦ The quality of the cosmid DNA is poor.	♦ The cosmid DNA should not be contaminated with E. coli DNA or endotoxins. Re-purify the cosmid, possibly on CsCl gradient.
	♦ The transfection was not efficient.	♦ Use new or freshly-made transfection reagents. If you followed our CaPO4 transfection protocol, pay attention to the following points: ♦ Use low-passage 293 cells. ♦ Prepare fresh HBS 2X rather than frozen aliquots. pH = 7.0 is very important. ♦ Wash the cells with DMEM-FBS about 8 hours after transfection. ♦ Monitor the transfection efficiency using the AdβGal DNA provided in the kit.
	♦ Your virus could be unstable or difficult to construct.	♦ Calculate the length of your recombinant virus, taking into account the deletions and insertions you made. The length should not exceed 37.8 kb. ♦ Keep culturing the dishes that were not agar-overlaid for a maximum of 3 weeks, splitting the cells when necessary. Some viruses take longer to propagate.
♦ **Do not get virus plaques in the dishes covered with agar, but well in those kept**	♦ Agar was too hot when poured and it killed the cells. In this case, the	♦ Repeat the plaque assay using the crude viral extract obtained from the

in liquid medium.	untransfected cells that were overlaid should have died too.	transfected dish that was kept in liquid medium (use dilutions: 10^4 to 10^8).
♦ **Get virus but with an unexpected genome structure.**	♦ The desired virus is not viable and the transfected DNA recombined to generate a mutant virus. This can be due to the size of your expression cassette exceeding the transgene capacity of the virus, or to the expression of your transgene which is toxic for the helper cells or incompatible with virus replication.	♦ Calculate the length of your recombinant virus, taking into account the deletions and insertions you made. If the length of your recombinant virus exceeds 37.8 kb, reduce the size of your insertions, or use a virus backbone that accommodates larger inserts. ♦ Use an inducible expression system for your transgene.
♦ **Get virus but no transgene expression.**	♦ The expression cassette cloned into the shuttle plasmid is inactive. The promoter, cDNA, or polyA signal was altered during the cloning process.	♦ Transfect the shuttle plasmid into a reporter cell line (such as 293 cells) and analyze transgene expression.
	♦ Your virus is unstable.	♦ Analyze transgene expression with other virus clones. ♦ Consider using an inducible expression system for your transgene.
	♦ Your recombinant virus is functional but it cannot infect your reporter cell line.	♦ Infect your reporter cell line efficiently with an adenovirus expressing a reporter gene (e.g. β-galactosidase) as control.

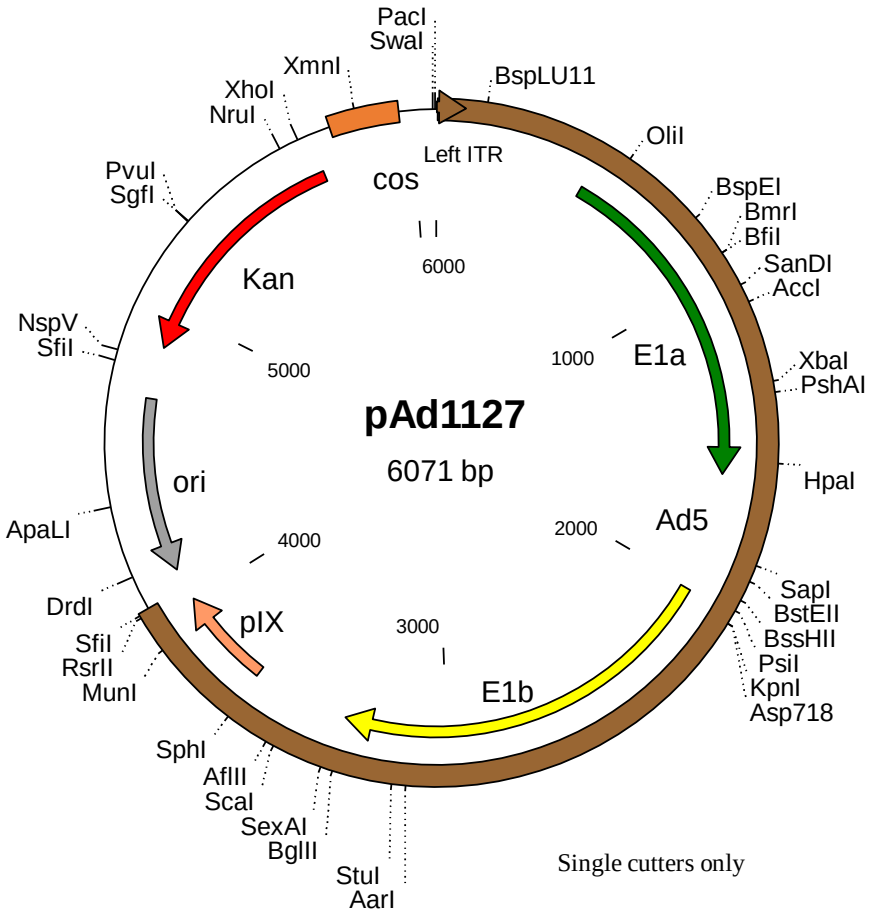
6 SHUTTLE PLASMID INFORMATION

pAd1127

6,071 base pairs - Sequence available at www.od260.com

pAd1127 is a plasmid designed for constructing recombinant adenovirus vectors, in combination with the AdenoQuick2.0 plasmids (pAd1128, pAd1129, pAd1130, and their derivatives). The plasmid can be used to manipulate the E1a, E1b, and pIX coding regions. It contains *Pac*I and *Swa*I sites flanking the first 4031 base pairs from the Ad5 genome (including the left ITR and packaging signal, E1a/b regions, and the pIX coding region). The sequences encompassing the kanamycin-resistance gene, the λ cos site, the adenovirus sequences are flanked by two SfiI restriction sites, which generate non-symmetrical sticky ends suitable for directional cloning.

Feature	Coordinates	Source
left ITR	5-107	Ad5
E1a (primary transcript)	502-1634	Ad5
E1b (primary transcript)	1703-4035	Ad5
pIX	3613-4035	Ad5
origin of replication	4699-4113	pUC19
Kan^r	5689-4877	*Tn903*
Cos	5753-5962	Phage λ

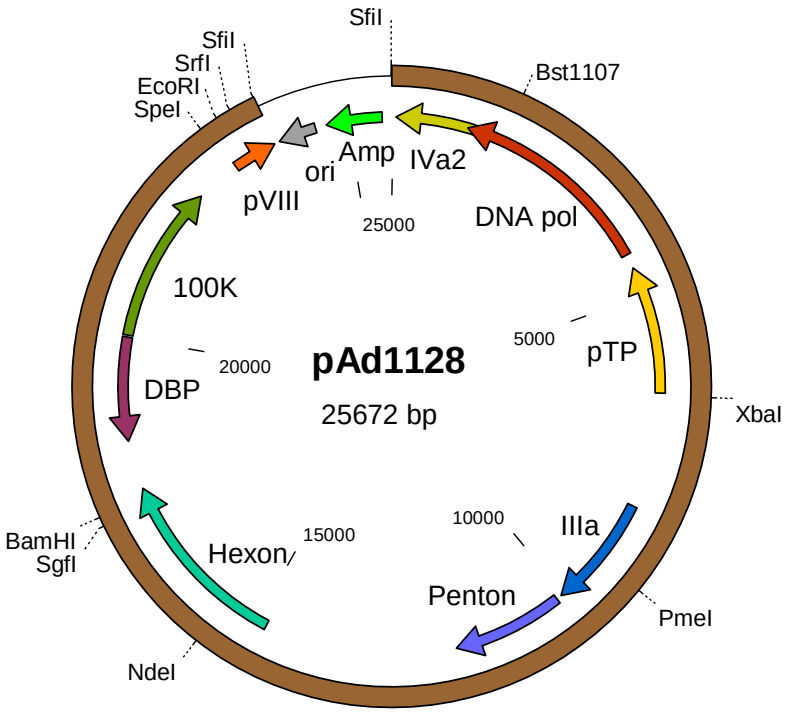


pAd1128

25,672 base pairs Sequence available at www.od260.com

pAd1128 is a plasmid designed for constructing recombinant adenovirus vectors, in combination with the AdenoQuick2.0 plasmids (pAd1127, pAd1129, pAd1130, and their derivatives). It contains the sequence encompassing psn 4033-27868 in 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. pAd1128 includes among others the complete ORFs encoding IVa2, DNA polymerase, pre-terminal protein, IIIa, penton, hexon, DNA-binding protein, 100K, and pVIII. The adenovirus sequences are flanked by two SfiI sites, which generate non-symmetrical sticky ends suitable for directional cloning.

Feature	Coordinates	Source
IVa2 (orf)	1423-65	Ad5
DNA pol (orf)	4341-1171	Ad5
pTP (orf)	6518-4557	Ad5
L1-IIIa (orf)	8292-10049	Ad5
Penton (orf)	10130-11845	Ad5
Hexon (orf)	14816-17674	Ad5
DBP (orf)	24032-22443	Ad5
100K (orf)	20035-22458	Ad5
pVIII	23148-23831	Ad5
origin of replication	24497-23910	pUC19
Amp^r	25528-24668	*Tn3*



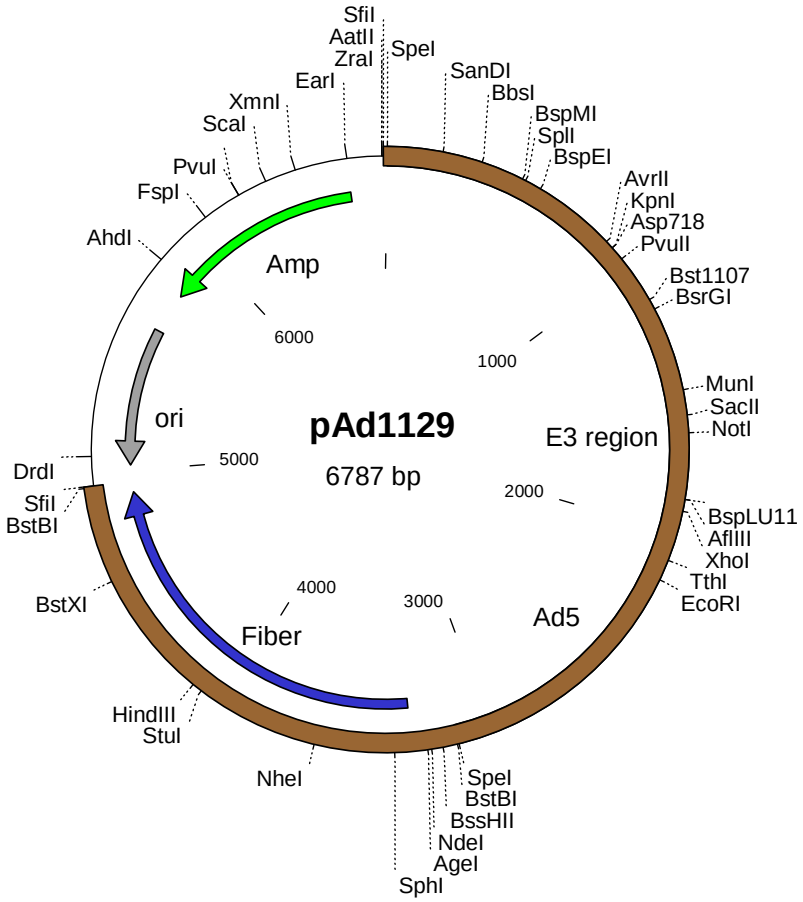
pAd1129

6,787 base pairs

Sequence available at www.od260.com

pAd1129 is a plasmid designed for constructing recombinant adenovirus vectors, in combination with the AdenoQuick2.0 plasmids (pAd1127, pAd1128, pAd1130, and their derivatives). It contains the sequence encompassing psn 27885-32795 in the Ad5 genome. pAd1129 includes all E3 genes, and the fiber coding region. The adenovirus sequences are flanked by two SfiI sites, which generate non-symmetrical sticky ends suitable for directional cloning.

Feature	Coordinates	Source
E3	~ 10- 3125	Ad5
Fiber (orf)	3170-4927	Ad5
origin of replication	5612-5024	pUC19
Amp^r	6643-5783	*Tn3*



pAd1130

10,059 base pairs

Sequence available at www.od260.com

pAd1130 is a plasmid designed for constructing recombinant adenovirus vectors, in combination with the AdenoQuick2.0 plasmids (pAd1127, pAd1128, pAd1129, and their derivatives). It contains the sequence encompassing psn 32801-right end in the Ad5 genome, including the right ITR and the entire E4 region. The right ITR is flanked with PacI and SwaI sites. The E4 region is terminated with two SfiI sites, which generate non-symmetrical sticky ends suitable for directional cloning. The plasmid contains a 5 kb stuffer made from scrambled phage λ DNA. This stuffer increases the size of the ligation product of pAd1127, pAd1128, pAd1129, and pAd1130 so that it can be packaged efficiently into phage λ.

Feature	Coordinates	Source
right ITR	7441-7543	Ad5
E4	4409-7440	Ad5
Scrambled stuffer DNA	9374-4324	phage λ
origin of replication	8699-9372	pUC19
Amp^r	7694-8554	*Tn3*