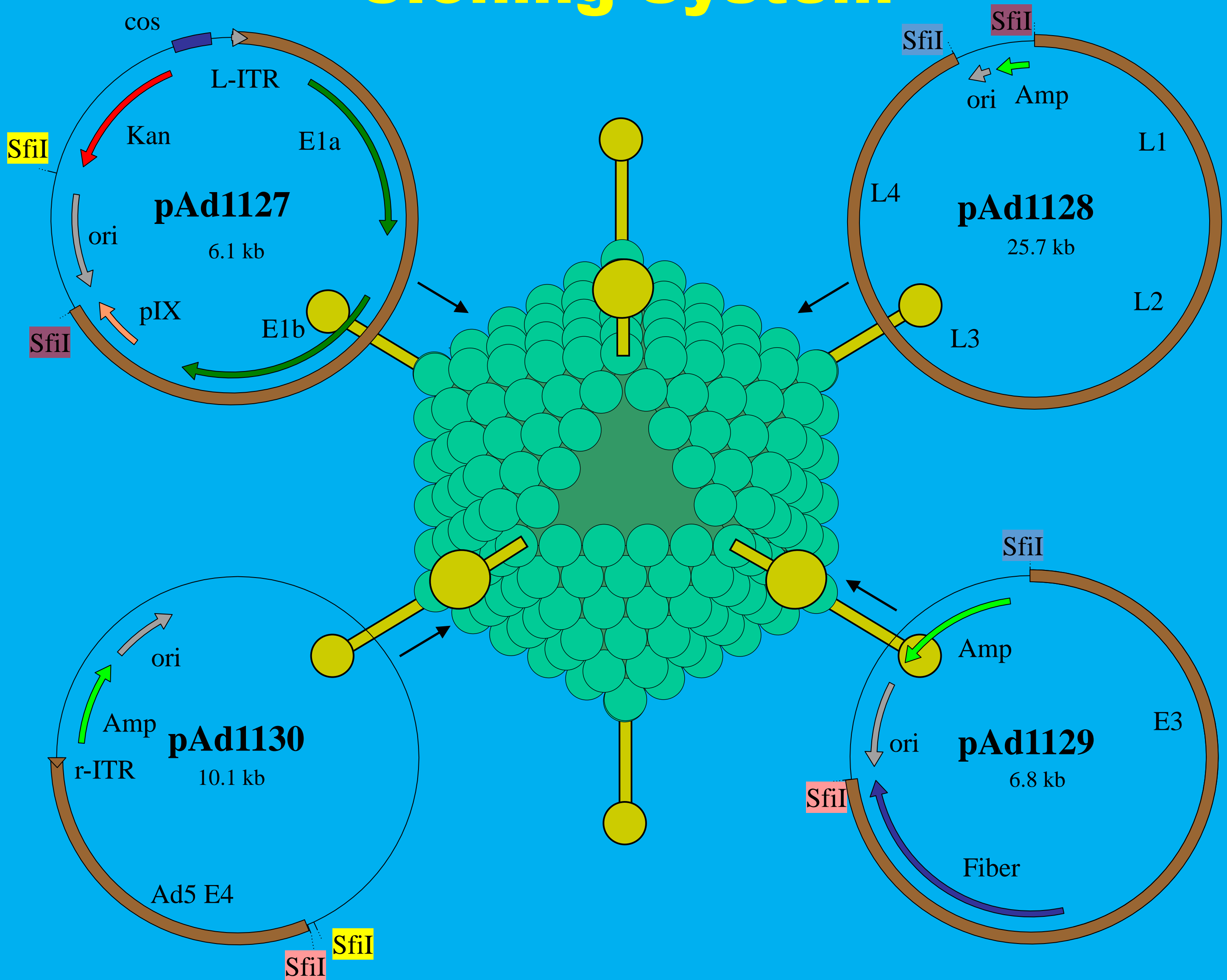
OD260 Inc.

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. 14).
- Become familiar with the different techniques used, especially those related to the manipulation of adenovirus (p. 15).
- 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 (https://osp.od.nih.gov/wp-content/uploads/NIH_Guidelines.htm).

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License Agreement

The purchased kit consists of the Materials identified in Section 2 (p. 13 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:

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- 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.
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- This agreement is between the Recipient and OD260 Inc. but also applies to members of the Recipient's direct research staff.

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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. These 4 shuttle plasmids can be manipulated independently using the method of your choice. You can also use one of the many derivatives that are already available. Make sure to visit our web site (https://od260.com/product/adenoquick-2.0-kit) for an updated list of AdenoQuick2.0 plasmids.

Once the shuttle plasmids are made, the reconstitution of the genome of your recombinant virus can be performed using 2 methods: lambda packaging or electroporation.

The construction of the cosmid via lambda packaging is very straightforward: mix the 4 plasmids, digest them with SfiI (30 min.), ligate them to each other (same tube, 1 hour), package the ligation products into phage λ (30 min.), infect E. coli with the packaged phage λ (30 min), plate the bacteria on a petri dish and incubate overnight.

The entire process is extremely efficient. It takes advantage of 4 features:

1. The SfiI recognition site (GGCCNNNN/NGGCC) is an 8-bp interrupted palindrome, which should be found statistically less often in DNA than most other restriction sites.

2. SfiI is a thermophile enzyme that works optimally at 50 °C. The activity of the enzyme is less than 10% at room temperature. This enables ligating SfiI-generated DNA fragments with each other in presence of the enzyme without the need for agarose gel, spin column clean-up or phenol-chloroform extraction.

3. The nature of the nucleotides making the cohesive ends can be arbitrarily chosen. The SfiI sites present in the 4 shuttle plasmids are therefore all different. They generate unique non-palindromic sticky ends that enable directional ligation between the four DNA fragments of interest.

4. Finally, the phage λ packaging method selects clones containing full-size genomes because phage λ packages DNA's ranging from 39 to 54 kb.

The construction of the cosmid via electroporation requires a little bit more hands-on time, but is also very efficient: digest the 4 shuttle plasmids individually with SfiI, purify the DNA fragments of interest on agarose gel, ligate them to each other, transform E. coli by electroporation, plate the bacteria on a petri dish and incubate overnight.

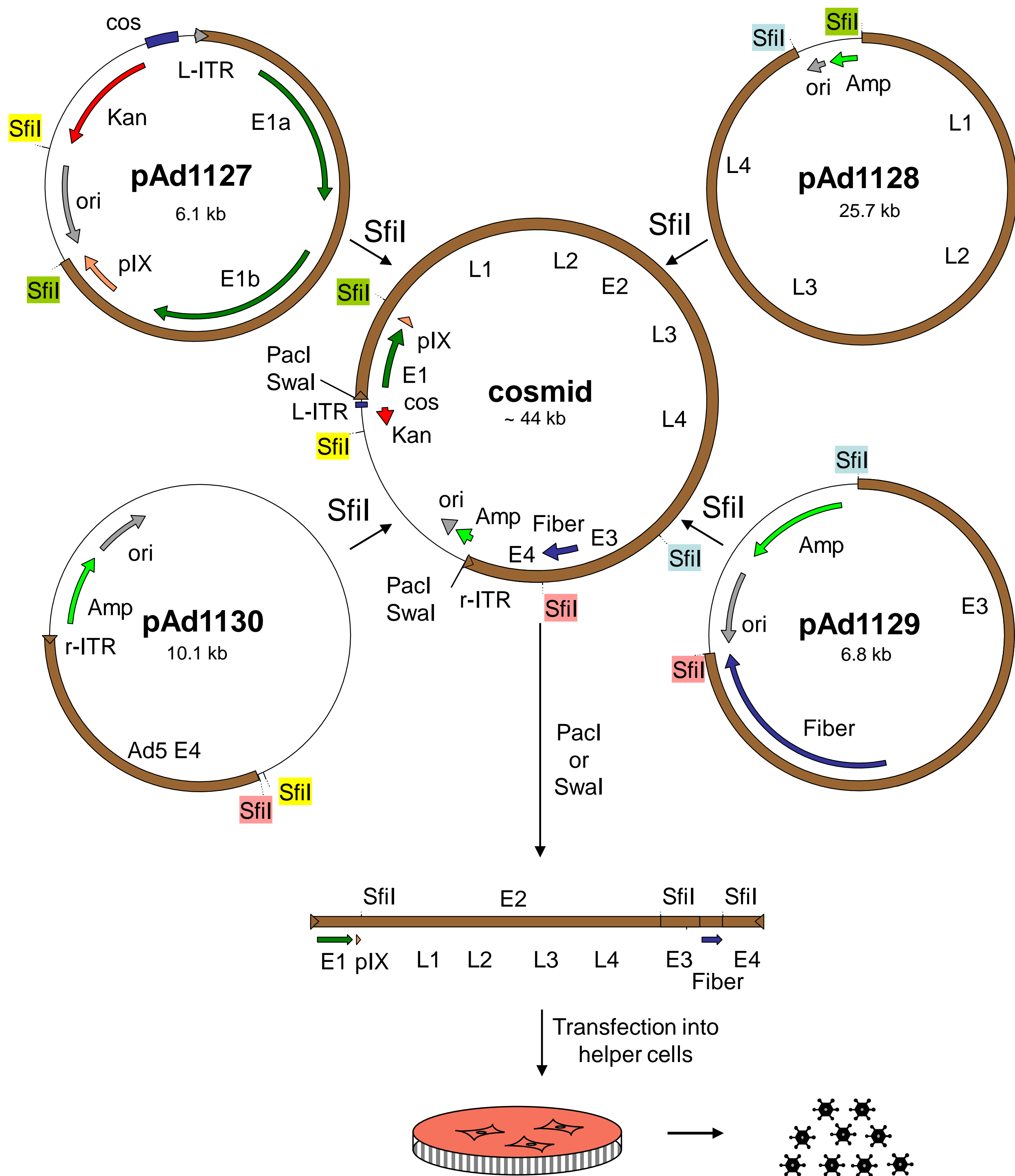

Figure 1: Overview of the AdenoQuick2.0 cloning system. The first step consists in modifying one or several of 4 shuttle plasmids (or derivatives) according to your needs: pAd1127 for the left ITR, packaging signal, E1 region, and pIX; pAd1128 for the E2 region and most late genes; pAd1129 for the E3 region and fiber; pAd1130 for E4 region and the right ITR. The 4 shuttle plasmids are then digested with SfiI, 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.

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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. Several E3 deletions are available, the most common being the 1.9 kb XbaI and 2.7 kb BglII deletions.

- **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.

- **Multigenic viruses**, containing two or more expression cassettes, for instance 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

  Multigenic viruses have several advantages over using several 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. However, much care must be given in the design of a multigenic vector: adenovirus has a very compact genome and modifying one region can affect one or several virus functions.

- **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 inserting 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 phage λ 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 E. coli* strains such as OD101, 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, this method is so efficient in generating the cosmid that a minimum number of E. coli clones needs to be analyzed.
    - The process of digesting the shuttle plasmids with SfiI, ligating them to each other, packaging the ligation products into phage lambda heads, infecting E. coli, and plating the bacteria on petri dishes takes less than 3 hours.
    - Cosmid DNA 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 #	Storage Temperature
pAd1127 or derivative	Shuttle plasmid E1 region + pIX	20 µg	www.od260.com	-20 °C
pAd1128 or derivative	Shuttle plasmid E2 region + late genes	20 µg	www.od260.com	-20 °C
pAd1129 or derivative	Shuttle plasmid E3 region + Fiber	20 µg	www.od260.com	-20 °C
pAd1130 or derivative	Shuttle plasmid E4 region	20 µg	www.od260.com	-20 °C
Cosmid Construction Kit, including:	Adenovirus cosmid construction via lambda packaging	10 reactions	R-1002	
➢ SfiI enzyme	Shuttle plasmid digestion	30 µL	R-1008	-20 °C
➢ SfiI 10x buffer	Shuttle plasmid digestion	200 µL	R-1009	-20 °C
➢ Lambda Packaging extract	Packaging the ligation products into phage lambda heads	50 µL	R-1005	-70 °C
➢ SM medium	Stopping the packaging reaction	1 mL	R-1004	-20 °C
➢ Maltose/$MgSO_4$	Preparing lambda-competent cells	700 µL	R-1023	-20 °C
➢ E. Coli OD101	Cosmid construction and amplification	200 µL	R-1006	-70 °C
➢ Amp/Kan antibiotic mix	Selection of colonies on Petri dishes	800 µL	R-1007	-70 °C
Cosmid Construction Kit, including:	Adenovirus cosmid construction via electroporation	10 reactions	R-1003	
➢ SfiI enzyme	Shuttle plasmid digestion	30 µL	R-1008	-20 °C
➢ SfiI 10x buffer	Shuttle plasmid digestion	200 µL	R-1009	-20 °C
➢ Electro-competent E. Coli OD101	Cosmid construction and amplification	2 x 20 µL	R-1024	-70 °C
➢ Outgrowth medium	Growing cells after electroporation	3 x 1 mL	R-1025	-20 °C
➢ Amp/Kan antibiotic mix	Selection of colonies on Petri dishes	800 µL	R-1007	-70 °C
Ad-ßGal DNA or Ad5-GFP DNA	Control for virus rescue by transfection	10 µg	ZC-01 or ZC-02	-70 °C
Handbook			on line	

3 BIOSAFETY INFORMATION

3.1 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.

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 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 TECHNICAL SKILLS

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.

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- 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 REAGENTS PROVIDED BY THE USER

Cosmid construction, per construct:

- 60 mL LB Lennox
- 2 petri dishes, each with ~ 20 mL LB Lennox/agar
- 5 µL chloroform
- 1 µL T4 DNA ligase
  - ☺ We recommend NEB # M0202S, 400,000 u/mL.
- Reagents for plasmid purification via the alkaline lysis method (P1/P2/P3).
- TE pH 7.5 (10 mM Tris-HCl, 1 mM EDTA, pH 7.5)
- SwaI (NEB # R0604S) or PacI (NEB # R0547S)

Virus rescue

- 293 cells (ATCC CRL-1573)
- DMEM/High Glucose (+ 4500 mg/L high glucose, + 4 mM L-glutamine, no sodium pyruvate; Hyclone # SH30022.02), supplemented with 10% Fetal Bovine Serum (not heat inactivated),100 u/mL Penicillin and 100 µg/mL Streptomycin.
- PBS 1x
- Trypsin-EDTA (0.25% Trypsin – 1 mM EDTA in PBS 1x)
- If you use the calcium-phosphate transfection method:
  - $CaCl_2$ 2M, 0.2 µm filter-sterilized
  - NaCl 5 M
  - Hepes 1 M, pH 7.3
  - Na_2HPO_4 (or NaH_2PO_4) 0.5 M

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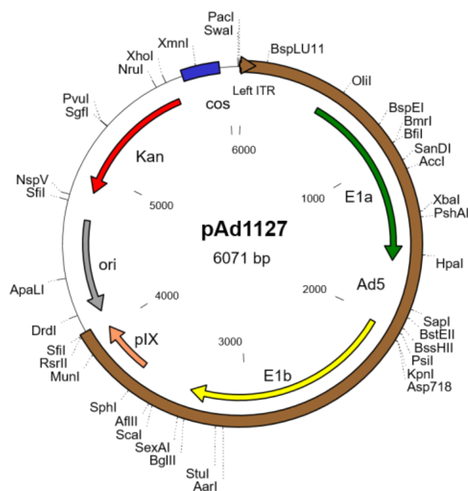
4.2 SHUTTLE PLASMID CONSTRUCTION

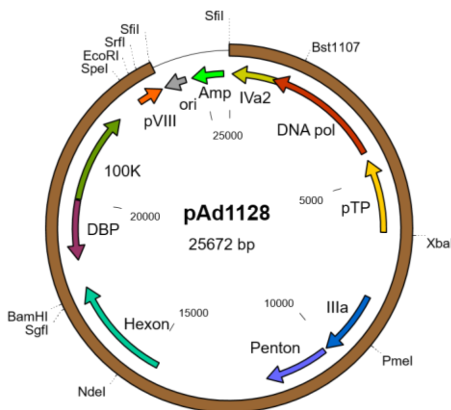
The first step towards the generation of your adenovirus vector is the construction of shuttle plasmids. There are 4 series of shuttle plasmids. You will need one plasmid from each series to construct your vector.

pAd1127 Series



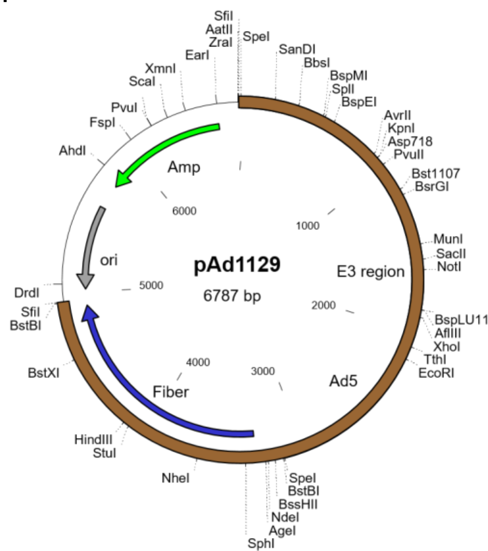
pAd1127 contains the left-end Ad5 sequence including the left ITR, packaging signal, the entire E1 region and the pIX gene. Many derivatives exist already, which differ by the packaging signal (repeats A1-A5 or A1-A7), the extent of the E1 deletion (starting at psn 350 or 450 in the Ad5 genome, and ending at psn 3300 or 3500), the presence of empty expression cassettes (CMV, RSV…), mutations in the E1A and E1B genes etc…

pAd1128 Series



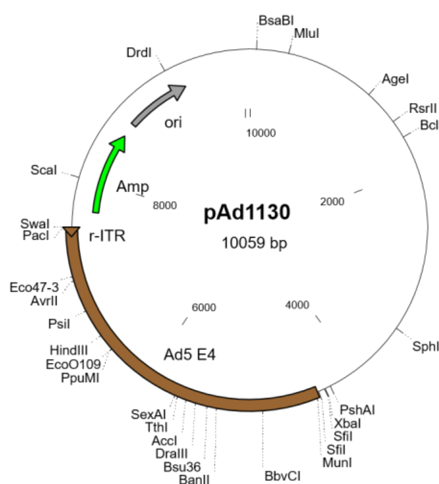
pAd1128 contains the Ad5 E2 region (DNA polymerase, terminal protein, DNA-binding protein) and most of the late genes (L1, L2, L3, L4).

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 your choice of recombinant DNA techniques.

☺ You can use any method: standard restriction digestion/ligation, recombination (Gateway, recombineering…), synthetic mini-genes, gBlocks, Gibson assembly, In-Fusion cloning, etc…

☺ Except for specific applications such as vaccine or oncolytic vectors, each transgene that you insert into the virus genome should have his own promoter and poly(A) signal. Do not rely on the adenovirus transcriptional elements for strong constitutive expression.

☺ If you are inserting an expression cassette controlled by a non-constitutive (e.g. tissue-specific, or inducible) promoter in place of the E1 region, you might want to orient it towards the left end of the adenovirus genome. 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. Keeping your promoter as far away from these E1A enhancers as possible, will minimize their interference.

- Verify the integrity of your shuttle plasmid by restriction analysis and/or sequencing.

4.3 ADENOVIRUS 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 can be done in 2 ways:

(1) Ligating the SfiI-digested shuttle plasmids with one another, packaging the ligated DNA into phage lambda phage heads and infecting competent E coli.

(2) Purifying the SfiI fragments of interest on agarose gel, ligating them with one another and transforming E coli by electroporation with the ligation products.

Both methods are very efficient. Method (1) requires less hands-on time.

4.3.1 METHOD 1: LAMBDA PACKAGING

Method (1) is performed in 4 steps in 3-5 hours:

1. Digesting the pAd1127, pAd1128, pAd1129, and pAd1130 plasmid derivatives with SfiI,
2. Ligating the SfiI-digested plasmid with each other,
3. Packaging the ligation products into phage λ,
4. Infecting E. coli with the packaged phage λ.

This method is very efficient, even if it involves a 4-DNA 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 all bacterial clones will carry the correct cosmid.

- **Preparation of λ-Competent *E. coli* (day -1 and day 1)**

□ On the day before the actual cosmid construction, streak *E. coli* OD101 from the glycerol stock provided in the kit (vial with grey cap) on a LB plate (no antibiotic). Incubate overnight at 37 °C.

□ On the morning of the cosmid construction day, add 2 mL LB + 60 µL $MgSO_4$/maltose solution (vial with yellow cap in the kit) in a sterile 10-mL culture tube.

□ Inoculate with one *E. Coli* OD101 colony.

- □ Grow at 37°C for 6-8 hours until the culture appears cloudy. Do not overgrow. Chill the cells on ice, and measure the cell density by absorbance at 600 nm (Abs600).

- □ Adjust the cell density to Abs600 =1 by diluting with LB.

➢ SfiI digestion (day 1)

- □ Digest 0.5 µg plasmid DNA from each series (pAd1127/28/29/30, thus 2 µg in total) in a single vial in a 25 µL volume with 1.5 µL SfiI.

- □ Incubate at 50 °C for 30 min.

- □ Chill on ice for 1 min.

  ☺ SfiI enzyme and 10x buffer are provided in the kit (vials with blue and white caps, respectively).

  ☺ SfiI 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.

  ☺ What if the heterologous sequence that you are inserting in a shuttle plasmid contains a SfiI site? If that extra SfiI site generates sticky ends that are different from the sticky ends generated by the other SfiI sites already present in the shuttle plasmids, the cloning efficiency will remain very high. If the sticky ends are identical to one of those generated by the other SfiI sites, the cloning efficiency will decrease. You might have to analyze more clones, but you will still be able to obtain the correct cosmid.

➢ DNA ligation

- □ Mix the following:

  8 µL SfiI-digested plasmid mix
  1 µL 10x T4 DNA ligase buffer
  1 µL T4 DNA ligase (400 u)

  ☺ Recommended T4 DNA ligase: New England Biolabs # M0202S, 400,000 units/mL

- □ Incubate at room temperature for 1 hour.

➢ Packaging

□ Add 2 µL ligation reaction to a microcentrifuge tube.

□ Thaw the λ packaging extract (vial with green cap in the kit), keeping it on ice as much as possible. Once thawed, add immediately 5 µL packaging extract to the ligation reaction. Mix by pipetting in/out once. Avoid making bubbles and do not spin down.

□ Incubate the packaging reaction at 30 °C for 30 min.

□ Add 100 µL SM buffer (vial with orange cap in the kit) and 5 µL chloroform. Mix vigorously for a few seconds by snapping the tube with your finger until white debris appear. Centrifuge for 15 sec., transfer the supernatant to a new tube (avoid the chloroform) and keep on ice until you are ready for the infection.

☺ Keep the λ packaging extract (vial with green cap) on ice at all times. Once you are done setting up the packaging reaction, quickly freeze the remainder packaging extract in liquid N_2 in order to preserve its activity as much as possible.

☺ Store the packaged phage extract at 4°C. It will be stable for a few months.

➢ Infection

□ Mix 60 µL packaging reaction with 60 µL diluted competent OD101 cells in a microcentrifuge tube. Avoid making bubbles and do not spin down.

□ Incubate for 30 min. at 37 °C.

□ Add 20 µL Amp/Kan antibiotic mix (vial with mauve cap in the kit) to the λ-infected cells, and spread on a LB petri dish.

☺ Use LB Lennox, since in presence of kanamycin, OD101 bacteria grow faster in low salt medium (Lennox: 5 g/L NaCl) than high salt medium (Luria Bertani, Miller: 10 g/L NaCl).

□ Incubate overnight at 37 °C.

4.3.2 METHOD 2: ELECTROPORATION

The construction of an adenovirus cosmid by electroporation is nothing else than the construction of a large plasmid by ligation of restriction fragments.

➢ SfiI digestion

☐ Digest the 4 shuttle plasmids (pAd1127, pAd1128, pAd1129, pAd1130 or derivatives, 5 µg each) with SfiI.

Typical Reaction:

5 µL DNA (1 µg/µL)
37 µL H2O
5 µL 10x SfiI buffer (reagent R-1009 in the kit, vial with white cap)
3 µL SfiI (reagent R-1008 in the kit, vial with blue cap)

☐ Incubate at 50 °C for 1 hour.

☐ Verify the completion of the digestions by agarose gel electrophoresis.

☺ SfiI 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.

☺ What if the heterologous sequence that you are inserting in a shuttle plasmid contains a SfiI site? If that extra SfiI site generates sticky ends that are different from the sticky ends generated by the other SfiI sites already present in the shuttle plasmids, the cloning efficiency will remain very high. If the sticky ends are identical to one of those generated by the other SfiI sites, the cloning efficiency will decrease. You might have to analyze more clones, but you will still be able to obtain the correct cosmid.

➢ Preparative agarose gel and DNA fragment purification

☐ Load the SfiI-digested plasmid DNAs on a 0.8% agarose gel in TAE buffer. Run the gel until the SfiI fragments are separated by 2-3 cm.

☐ Cut the SfiI fragments of interest out of the gel.

- ☐ Extract the SfiI fragments from agarose using your favorite method.

  ☺ We recommend a gel-melting method based on Sodium Iodide or Guanidine Thiocyanate, and followed by purification on a silica spin column.

- ☐ Verify the integrity and the yield of the purified DNA fragments by agarose gel electrophoresis. Estimate the relative concentrations of the various DNA fragments from their intensities on gel.

➢ DNA ligation

- ☐ Ligate equimolar amounts of the 4 purified SfiI DNA fragments with each other.

Typical Reaction:

2 µL SfiI-digested pAd1127 (or derivative)
2 µL SfiI-digested pAd1128 (or derivative)
2 µL SfiI-digested pAd1129 (or derivative)
2 µL SfiI-digested pAd1130 (or derivative)
1 µL 10x T4 DNA ligase buffer
1 µL T4 DNA ligase (NEB # M0202S, 400,000 units/mL)
Total = 10 µL

- ☐ Incubate at room temperature for 1 hour, or overnight at 4 ˚C.

➢ Electroporation

- ☐ Thaw the outgrowth medium in a 37 ˚C waterbath (vial with orange cap in the kit).
- ☐ Add 40 µL ddH2O to a microcentrifuge tube labeled “A”. Chill on ice.
- ☐ Chill a 2 mm-gap sterile electroporation cuvette on ice.
- ☐ Thaw the electro-competent cells (vial with yellow cap in the kit). Keep the vial on ice as soon as the cells are thawed.
- ☐ Add 1 µL ligation reaction to tube “A” containing 40 µL H2O.
- ☐ Add 3 µL electro-competent cells to tube “A”. Mix briefly to homogenize.

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☐ Transfer the contents of tube “A” to the bottom of the chilled electroporation cuvette. Immediately place the cuvette in the electroporation chamber of your device. Apply electric choc.

☺ Consult the instruction manual of your electroporation device for the correct settings.

☐ Immediately retrieve the cuvette from the device. Add 300 µL warm outgrowth medium at the bottom of the cuvette. Pipet in/out a few times to resuspend the cells. Transfer the cell suspension to a 10-mL sterile culture tube. Grow the cells for 45 min at 37˚C with shaking.

☐ Add 20 µL ampicillin/kanamycin (vial with mauve cap in the kit) to the cell suspension, then immediately plate the cells on a Petri dish with LB agar.

☺ Use LB Lennox, since in presence of kanamycin, OD101 bacteria grow faster in low salt medium (Lennox: 5 g/L NaCl) than high salt medium (Luria Bertani, Miller: 10 g/L NaCl).

☐ Incubate overnight at 37 ˚C.

☺ Re-freeze the vial of electro-competent cells quickly in liquid N2, then transfer to – 70˚C freezer. It will not lose its efficacy.

4.3.3 COSMID MINI-PREPS

☺ At this point you can store the Petri dishes at 4 °C, but we advise not to wait more than 2-3 days before growing the miniprep cultures. The fresher, the better.

➢ Small-scale bacterial cultures

☐ **Morning**: Prepare 4 x 10-mL culture tubes with 2 mL LB Lennox, each supplemented with 2 µL Amp/Kan antibiotic mix (vial with mauve cap in the kit).

☐ Inoculate each tube with a single colony that you obtained overnight.

☐ Grow at 37°C for 8-10 hours with shaking.

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

☺ Do not exceed 12 hours growth, otherwise the cosmid DNA yield will drop.

➢ Cosmid DNA Mini-preps

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

- □ Purify the cosmid DNAs using the alkaline lysis method. Resuspend the cosmid DNAs in 50 µL TE pH 7.5.

  ☺ Alkaline lysis method quick protocol:

  - Centrifuge 1.5 mL bacterial culture for 2 min @10,000 rpm. Discard the supernatant.
  - Resuspend the cell pellet in 150 µL Solution 1 (50 mM Tris-Cl, 10 mM EDTA, pH 8.0, 100 µg/mL RNase A)
  - Add 150 µL Solution 2 (1% SDS (w/v), 0.2 N NaOH). Incubate at RT for 3 min.
  - Add 150 µL Solution 3 (3 M Potassium acetate, pH 5.2). Shake vigorously 3-4 times, until white debris appear and no viscosity remains. Do not vortex.
  - Centrifuge at 14,000 rpm for 2 min.
  - Transfer the supernatant to a new tube. Add 920 µL EtOH.
  - Chill on ice for 10 min.
  - Centrifuge at 4°C for 15 min. Discard the supernatant as much as possible. Let the pellet air-dry.
  - Resuspend the pellet in 50 µL TE pH 7.5.

- □ Verify clone identity by restriction analysis (5 µL cosmid DNA per digestion).

  ☺ We suggest using HindIII at first. It will generate a restriction pattern that is easy to identify on a 0.8% agarose gel in TAE buffer. Verify the positive clones with 1 or 2 more enzymes that cleave inside your expression cassette.

4.3.4 COSMID MIDI-PREP

□ **Bacterial Culture (day -1, late evening)**: Inoculate a positive clone (100 µL mini-prep culture) into 40 mL LB supplemented with 40 µL ampicillin/kanamycin antibiotic mix provided in the kit (vial with mauve cap).

☺ We recommend that once you get the *E. coli* clones carrying your cosmid, you grow immediately the medium-scale bacterial cultures.

☺ A 40-mL culture should provide at least 30-40 µg cosmid DNA, which is enough for the virus recovery and characterization.

□ Harvest the cells at the end of the exponential growth phase, usually after 8-12 hours.

☺ 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.

☺ Now is a good time to make a glycerol stock of your clone for long-term storage at – 70°C (400 µL bacterial culture + 100 µL sterile glycerol).

□ 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), Wizard Purefection Plasmid prep midi kit (Promega), and the PureLink HiPure plasmid midiprep kit (Invitrogen). 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.

☺ Use 1 µg DNA if you digest it with an enzyme such as HindIII, which generates about ~10 fragments ranging from 1 to 8 kb.

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

However we recommend to isolate viral clones. The main reason is linked to virus stability. Your recombinant virus 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 laboratory practice to isolate and analyze several virus plaques.[*]

Viral crude lysates obtained directly from the dishes transfected with the plasmid DNA 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 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

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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 in *E. coli*.

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.

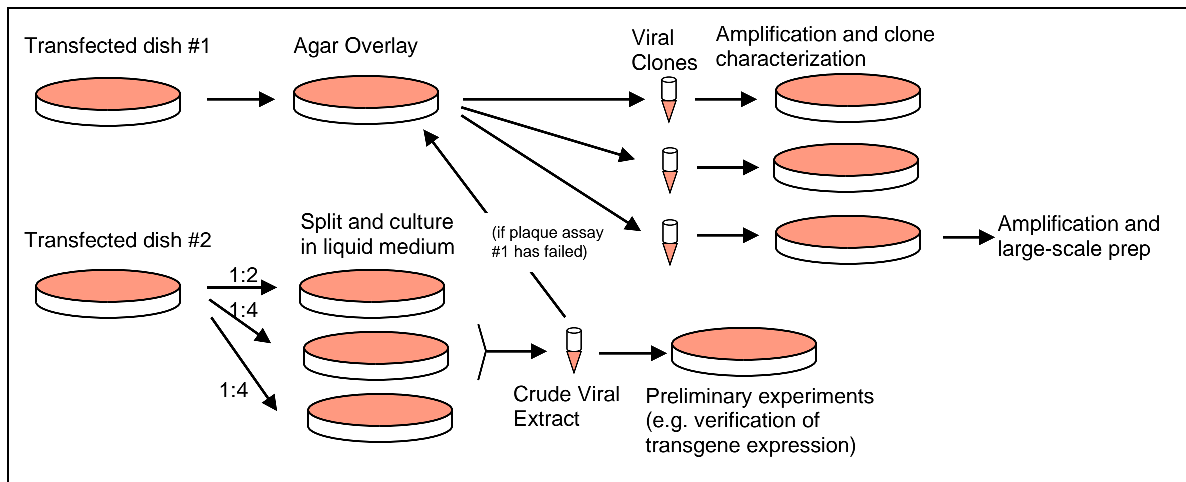
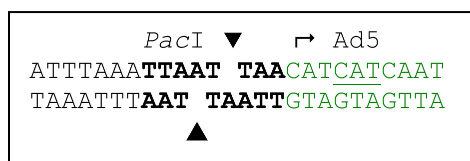


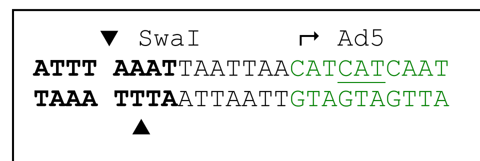
Figure 2: Proposed Flow-Chart to Isolate, Analyze and Amplify your Recombinant Virus.

4.4.1 COSMID DNA PREPARATION

☐ Digest 15 µg cosmid DNA with either *Pac*I or *Swa*I, whichever is not present in your gene of interest.

☺ The AdenoQuick system offers the choice between *Pac*I and *Swa*I for excising the adenovirus genome from the cosmid. 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.

- ☐ Verify the completion of the digestion and the quality of the DNA by agarose gel electrophoresis.

☺ 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.

- ☐ Precipitate the DNA with EtOH, centrifuge and resuspend the DNA pellet in 60 µL sterile TE pH 7.5 (⇒ ~0.25 µg/µL).
- ☐ Proceed to the transfection step.

4.4.2 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 15-cm dish containing 24 mL warm DMEM supplemented with 10% FBS and penicillin/streptomycin. Change the medium the next day. If necessary, split the cells 1:3 as soon as they reach confluence.

☺ Use <u>low-passage</u> 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 (+ 4500 mg/L high glucose, + 4 mM L-glutamine, no sodium pyruvate; Hyclone # SH30022.02), supplemented with 10% Fetal Bovine Serum (not heat inactivated), 100 u/mL Penicillin and 100 µg/mL Streptomycin
- PBS 1x
- Trypsin-EDTA (0.25% Trypsin – 1 mM EDTA in PBS 1x)

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

4.4.3 TRANSFECTION

The following protocol has been optimized for the calcium phosphate/DNA precipitation method, which is very efficient with 293 cells. Other methods such as lipofection can be used.

□ Early in the morning, split a newly confluent 15-cm dish of 293 cells into six 6-cm dishes at 30% confluence. By the end of the afternoon (8-10 hours later), the cells should be adherent and 30-50 % confluent.

☺ Plan to transfect 2 dishes per adenovirus construct. Include also a negative control (untransfected cells) and a positive control for the transfection. The positive control consists in transfecting 293 cells with a cosmid-purified adenovirus DNA expressing *E. coli* β-galactosidase or eGFP (AdßGal DNA cat # ZC-01; Ad5.CMVeGFP DNA cat # ZC-02). Those reporter adenoviruses could be useful in your future experiments.

□ 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 just before the 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 (in duplicate)*	*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 a 1-mL Pipetman. Do not swirl, put the cells straight back into the incubator.

□ Approximately 12 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 an appearance that can be mistakenly identified as a cytopathic effect caused by the virus.

4.4.4 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 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. Pellet 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

5% 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 appear, for up to 2 weeks. Feed the cells every 3-4 days with ¼ volume DMEM/10% FBS.

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

5 TROUBLESHOOTING

5.1 COSMID CONSTRUCTION

Observation	Possible Cause(s)	Comments & Suggestions
♦ **No colony recovered after >12 hours incubation at 37 °C**	♦ 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 16°C 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 30°C 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 +4°C 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 $MgSO_4$ 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 $CaPO_4$-DNA co-precipitation method, this should have been done 8-12 hours after the transfection.

Problem	Possible cause	Solution
♦ **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.

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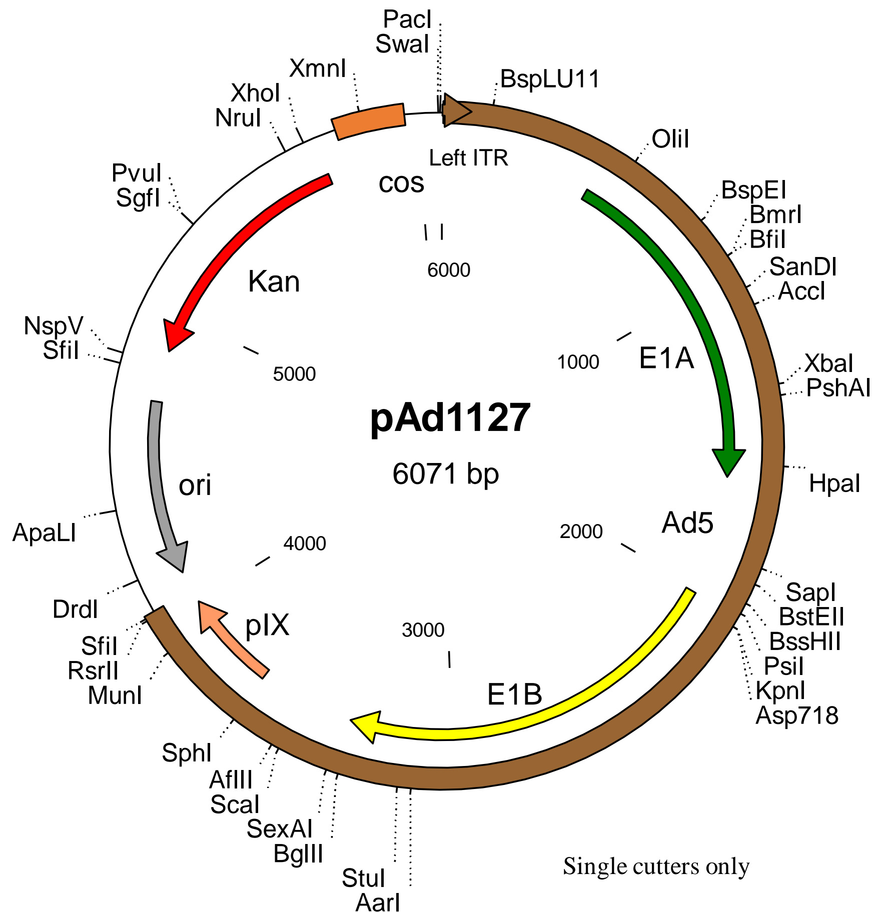
6 SHUTTLE PLASMID INFORMATION

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

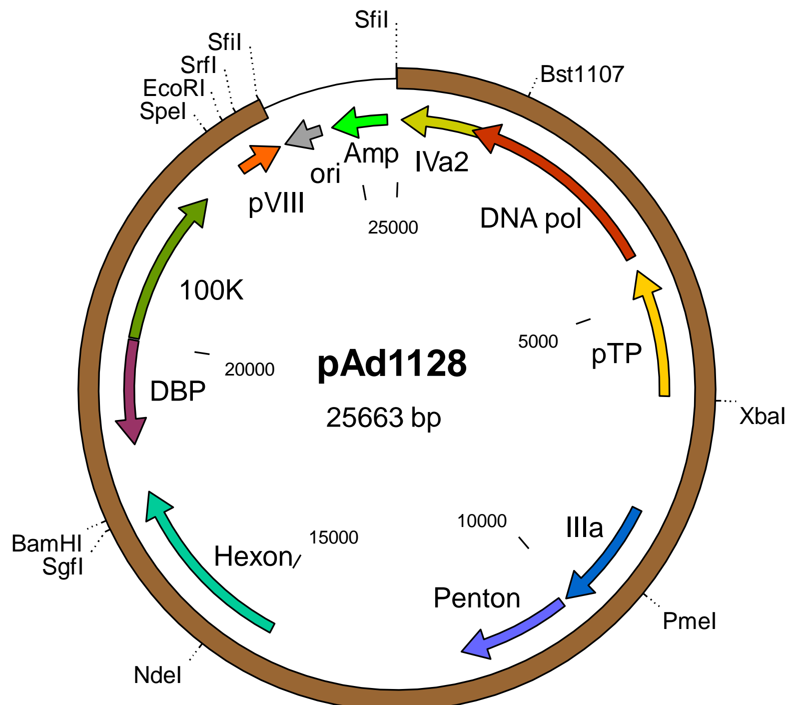


pAd1128

25,663 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 4032-27852 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*



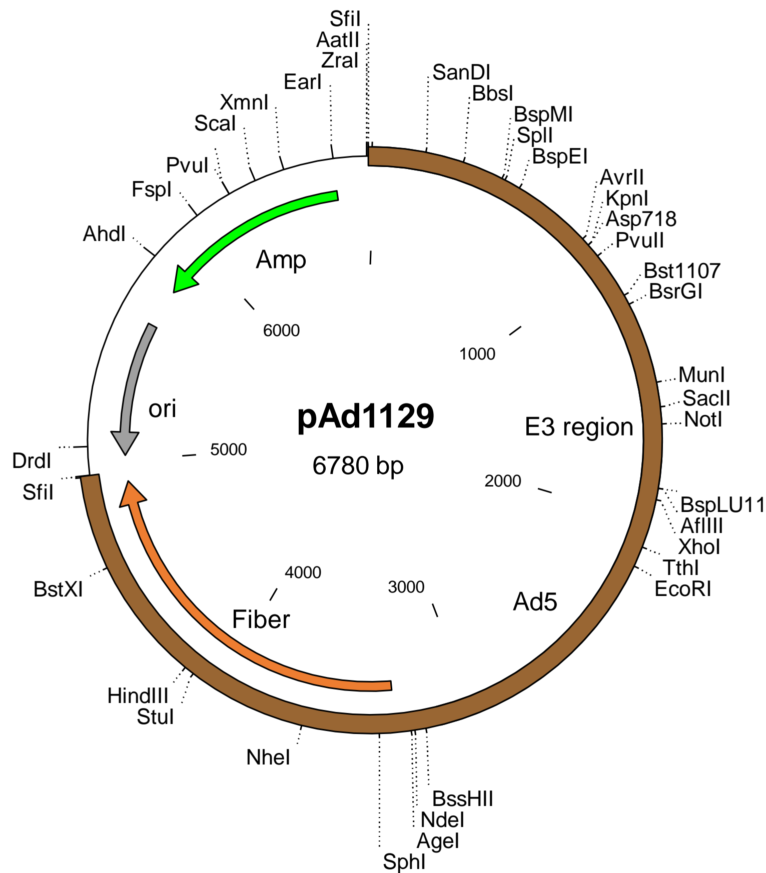
pAd1129

6,780 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*