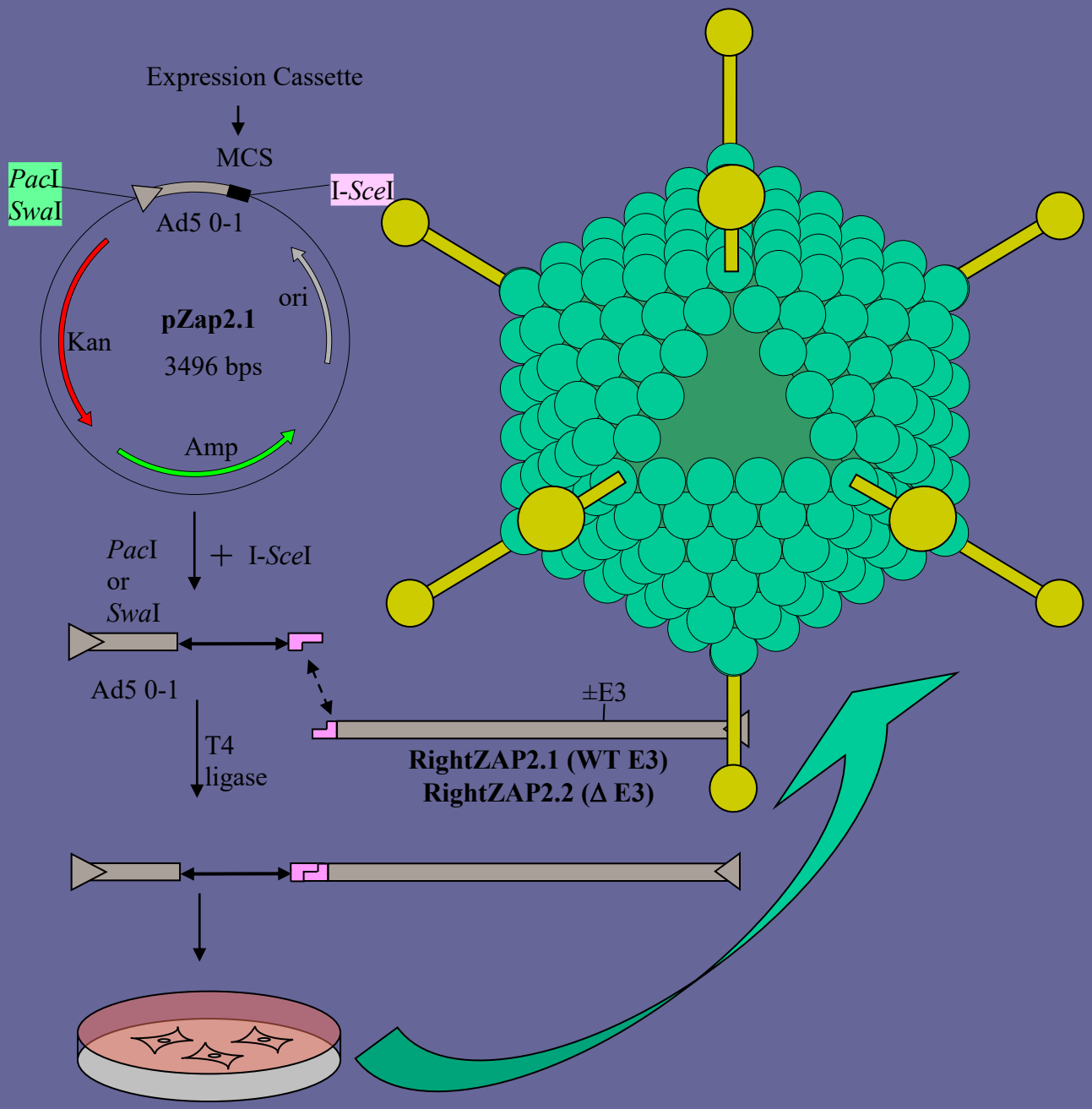
O.D.260 Inc.

The AdenoZAP™ Cloning System



Important Notice

Before opening the kit:

- Read our License Agreement (p. 3)
- Read the entire manual, especially the chapter “Safety Considerations about Adenoviruses” (p. 12).
- Become familiar with the different techniques used, especially those related to the manipulation of adenovirus.
- Inquire about regulatory rules for recombinant DNA with your institution or company. If none exists, refer to the NIH guidelines for research involving recombinant DNA molecules (http://osp.od.nih.gov/office-biotechnology-activities/biosafety/nih-guidelines)

License Agreement

The purchased kit consists of the Materials identified in Section G (p. 41 of this manual). In the course of carrying out experiments, it is anticipated that the Recipient will create various Derivatives of the Materials. Derivatives are defined as other materials including, without limitation, DNA, plasmids and viral vectors. By opening the kit and using these products, the Recipient agrees with the following:

1. The Recipient agrees to use the Materials and Derivatives solely for experimental purposes. These products will not be used, under any circumstances, in humans or for any human diagnostic or commercial purposes.
2. The Recipient agrees that the Materials and Derivatives will be used only at the Recipient's facilities and only by the Recipient or under the Recipient's direct supervision. The Recipient agrees to refrain from distributing or releasing samples or copies of the Materials or Derivatives to any third party.
3. The Recipient shall hold 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.
4. 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.
5. 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.
6. The Recipient agrees to comply with all laws and regulation for the handling and use of the Materials. He 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.
7. 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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A. The AdenoZAP™ Cloning System: Principle and Advantages

The AdenoZAP™ Cloning System is based on the *in vitro* ligation of two DNA molecules. The first molecule, which we refer to as the "left arm", contains the adenovirus left ITR, packaging signal and your expression cassette. The second DNA molecule, which we refer to as the "right arm", contains most of the adenovirus genome (from bp 3504 to the right end, with or without a 2.7 kb E3 deletion).

The left arm is obtained by cloning your expression cassette into a small shuttle vector, digesting the resulting plasmid with two endonucleases, and purifying the fragment of interest on agarose gel.

The right arm is provided in the kit. It originates from a bacterial cosmid rather than from virions, thereby eliminating the danger for a possible viral contamination. It will generate virus only after being ligated to the left arm that contains your expression cassette and the packaging signal.

We have designed the AdenoZAP cloning system in such a way that the ligation of both left and right arms is directional. Because of the non-symmetrical nature of the sticky ends generated by excising the left arm from the shuttle vector, the ligation of the left and right arms should generate only one type of viral DNA molecule. Consequently, a homogeneous virus population should be generated upon transfection of the helper cells. In addition, such ligation is performed to almost completion in less than an hour using an ordinary T4 DNA ligase.

The ligation product is directly transfected into a helper cell line such as 293 cells. Viral plaques usually appear 7 days after transfection, sometimes as early as 3 days.

Compared to other methods currently available, the AdenoZAP™ Cloning System has the following advantages:

- **Only one cloning step in *E. coli***
  The only cloning you have to perform in *E. coli* is inserting your expression cassette into a small shuttle vector.
- **No large plasmid (or cosmid) construction and purification**

The AdenoZAP™ cloning system - *version 1.x*

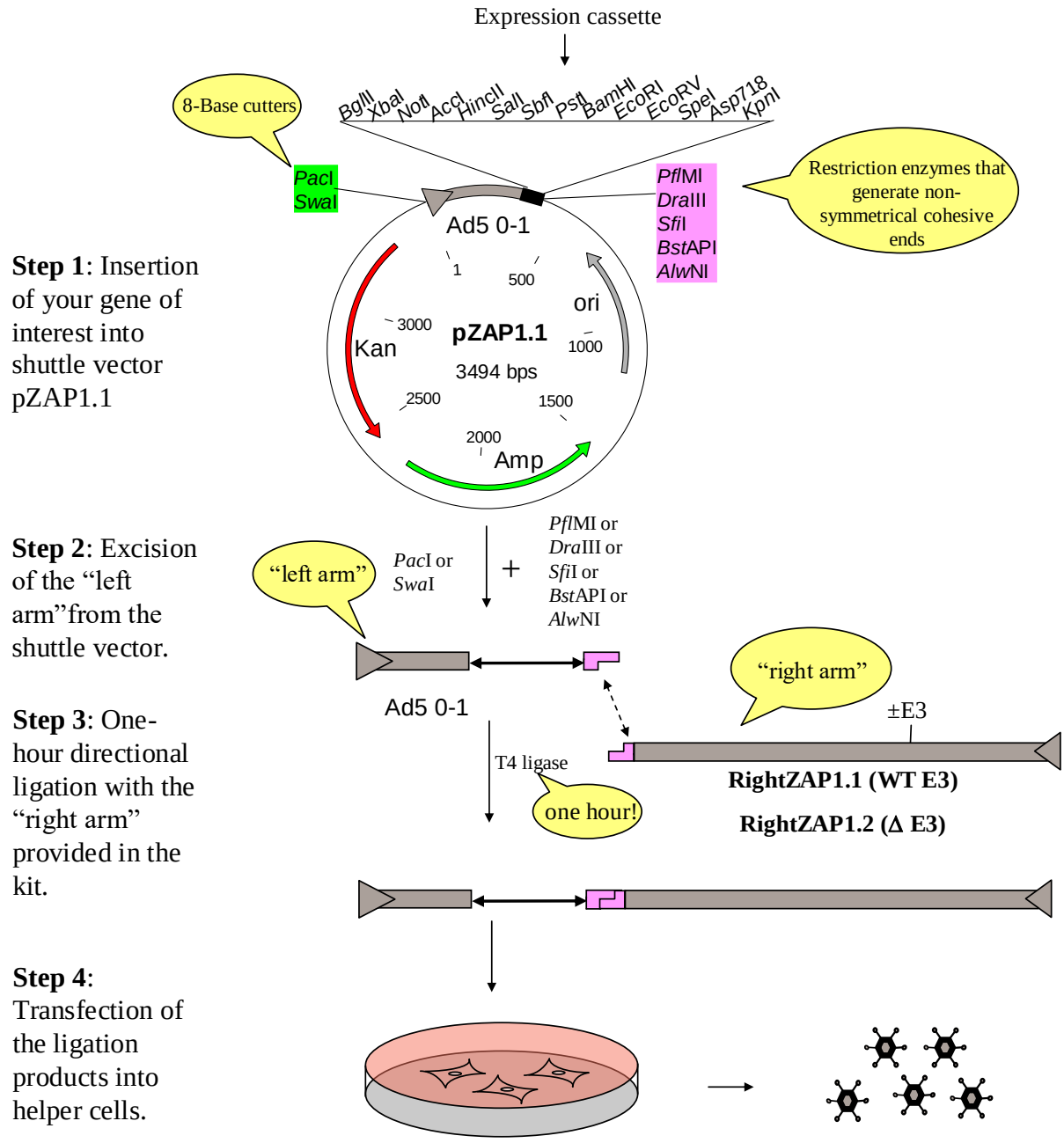
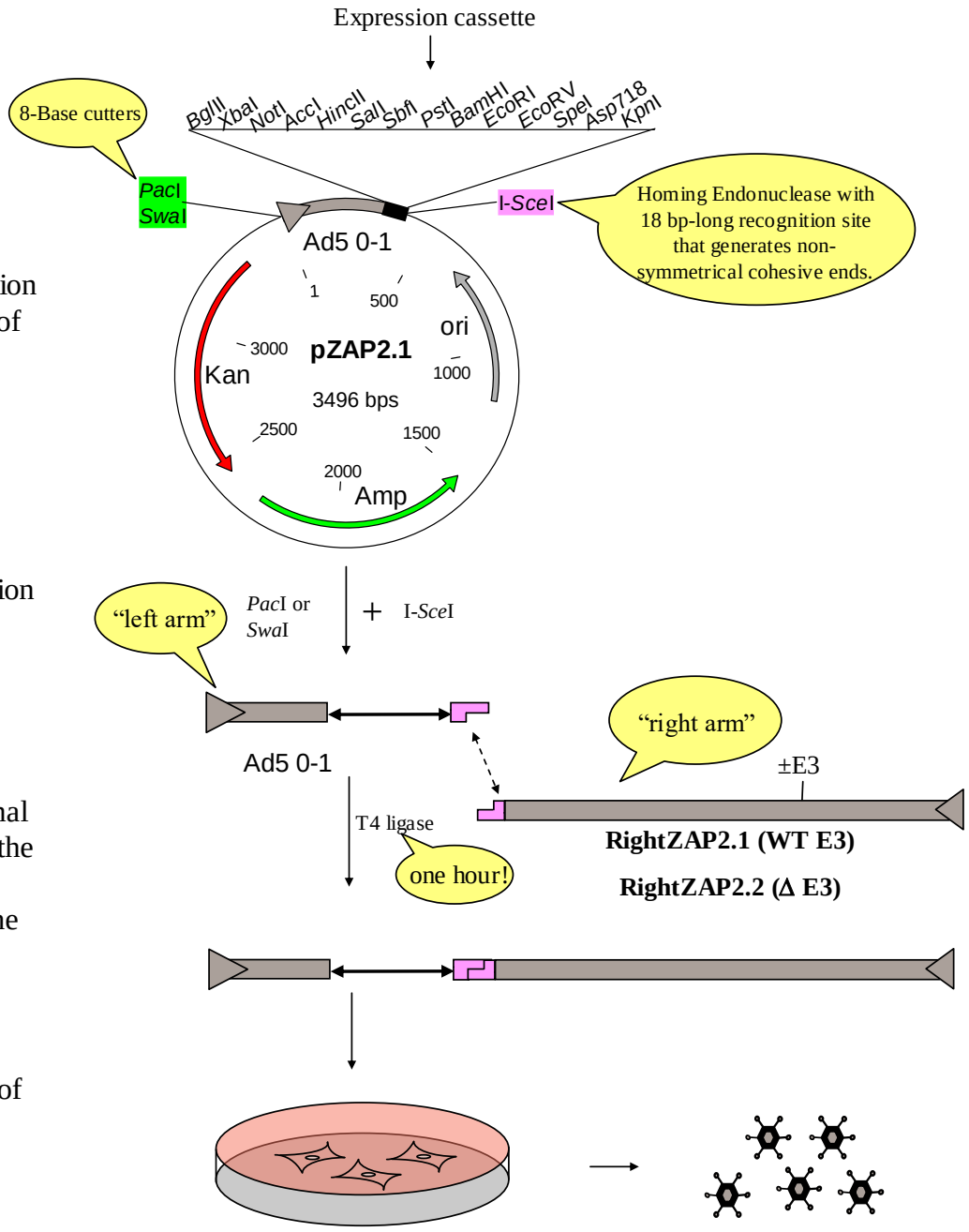


Figure 1: Construction of Adenovirus Vectors using the AdenoZAP Cloning System (Version 1.x)

The brown bar represents the adenovirus genome. The triangles indicate both left and right ITRs. Ad5 0-1: first map unit (mu) of the Ad5 genome (bp 1-353), including the left ITR and the packaging signal. The black rectangle indicates the multiple cloning site. The double arrow symbolizes the expression cassette. Pink L-shaped boxes highlight the non-symmetrical sticky ends. Kan: kanamycin-resistance gene (Tn 903). Amp: ampicillin-resistance gene. Ori: pUC19 origin of replication.

The AdenoZAP cloning system - *version 2.x*



Step 1: Insertion of your gene of interest into shuttle vector pZAP2.1

Step 2: Excision of the "left arm"from the shuttle vector

Step 3: One-hour directional ligation with the "right arm" provided in the kit

Step 4: Transfection of the ligation products into helper cells

Figure 2: Construction of Adenovirus Vectors using the AdenoZAP Cloning System (Version 2.x). The brown bar represents the adenovirus genome. The triangles indicate both left and right ITRs. Ad5 0-1: first map unit (mu) of the Ad5 genome (bp 1-353), including the left ITR and the packaging signal. The black rectangle indicates the multiple cloning site. The double arrow symbolizes the expression cassette. Pink L-shaped boxes highlight the non-symmetrical sticky ends. Kan: kanamycin-resistance gene (Tn 903). Amp: ampicillin-resistance gene. Ori: pUC19 origin of replication.

Constructing large plasmids or cosmids is not obvious for inexperienced researchers. In addition, purifying them in a quality suitable for transfection (i.e. without *E. coli* chromosomal DNA) is difficult using column chromatography and is better performed on CsCl gradients, which is time-consuming and hazardous.

- **No plasmid midi-prep or maxi-prep necessary**
  Although using a purified preparation of your shuttle plasmid maximizes the chances of getting your recombinant virus, it is not necessary. You can use mini-prep DNA: just clean it up after verifying the identity of your clone, digest it with the appropriate enzymes and purify the fragment of interest on agarose gel. A few micrograms plasmid DNA are usually sufficient to perform the whole procedure.
- **A versatile set of endonucleases available to generate the left arm**
  Two versions of AdenoZAP cloning system are available. In AdenoZAP1.x, two 8-base cutters (*Pac*I and *Swa*I) are available to digest your shuttle vector next to the left ITR, and four 6-base cutters (*Alw*NI, *Bst*API, *Dra*III, *Pfl*MI) and one 8-base cutter (*Sfi*I) are available to cut your shuttle vector downstream from your expression cassette. In AdenoZAP2.x, the same 8-base cutters (*Pac*I and *Swa*I) are available to digest your shuttle vector next to the left ITR, while an 18-base intron-encoded endonuclease (I-*Sce*I) is available to cut the shuttle vector downstream from your expression cassette. It is therefore likely that this system will be useful in a very large number of applications.
- **Fast**
  With only one cloning step in *E. coli*, a DNA digestion, gel purification and a 1-hour ligation before the transfection can be performed, the overall hands-on time is strongly reduced compared to most of the other methods currently available. If well organized, one can perform the DNA transfection into helper cells only 3 days after starting the cloning of an expression cassette into the shuttle vector and harvest the first virus plaques 3-7 days later.

B. Choosing the AdenoZAP Cloning Kit Suitable for your Application

The AdenoZAP™ cloning system exists in different versions and each version can be used in different ways. The following table will help you decide which one is the most appropriate to your application.

Table 1: Overview of the Various AdenoZAP Cloning Systems.

Kit	Shuttle Vector	Enzymes available for Left Arm Excision		Right Arm	E3 Region Status	Maximum Transgene Capacity
		Left End	Right End			
AdenoZAP1.1	pZAP1.1	*Pac*I, *Swa*I*	*Sfi*I, *Bst*API, *Dra*III, *Alw*NI, *Pfl*MI	RightZAP1.1	WT E3	5.2 kb
AdenoZAP1.2	pZAP1.1	*Pac*I, *Swa*I*	*Sfi*I, *Bst*API, *Dra*III, *Alw*NI, *Pfl*MI	RightZAP1.2	ΔE3	7.9 kb
AdenoZAP2.1	pZAP2.1	*Pac*I, *Swa*I	I-*Sce*I	RightZAP2.1	WT E3	5.2 kb
AdenoZAP2.2	pZAP2.1	*Pac*I, *Swa*I	I-*Sce*I	RightZAP2.2	ΔE3	7.9 kb

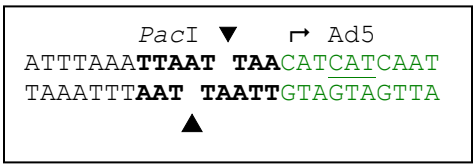
* *Swa*I is not available for AdenoZAP1.1 and 1.2, lots 0101.

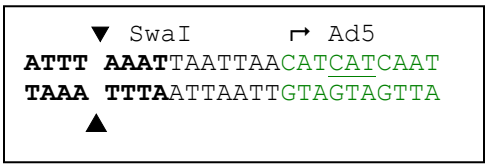
1. AdenoZAP1.x or AdenoZAP2.x?

Both the AdenoZAP1.x and AdenoZAP2.x cloning systems rely on endonucleases that generate non-symmetrical sticky ends with which directional ligation can be performed. The only difference between both systems is the nature of the enzymes available to excise the left arm from the shuttle vector pZAP. In pZAP1.1, the left arm can be excised using *Pac*I or *Swa*I on the left side, and either *Sfi*I, *Bst*API, *Dra*III, *Alw*NI or *Pfl*MI on the right side. *Pac*I and *Sfi*I are 8-base cutters while *Bst*API, *Dra*III, *Alw*NI and *Pfl*MI are 6-base cutters. In pZAP2.1, the left arm can be excised using *Pac*I or *Swa*I on the left side and I-*Sce*I on the right side. I-*Sce*I is an intron-encoded endonuclease that recognizes an 18-bp-long sequence within which minimal sequence degeneracy is tolerated. Although the pZAP1.1 offers a large variety of enzymes, pZAP2.1 is even more universal because of the rarity of the I-*Sce*I recognition site.

2. *Pac*I or *Swa*I?

The AdenoZAP system offers the choice between *Pac*I and *Swa*I for digesting pZAP at the border of the left arm. Both enzymes are 8-base cutters, thus they should be present at equal frequencies in DNA. The only difference is the position of the restriction sites relative to the start of the adenovirus left ITR. As illustrated below, *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.

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 3 days after transfecting *Pac*I- or *Swa*I-digested DNA into 293 cells.

3. WT E3 or Δ E3?

In the virus replication cycle, the expression of the E3 region helps the virus to evade the host immune system. This region is not essential for virus replication *in vitro* and therefore can be deleted in order to construct adenoviruses containing longer transgenes, up to 8.2 kb. However, in some applications, it might be desirable to retain or even increase the expression of some E3 products: for instance, the adenovirus "death protein" E3-11.6K, which facilitates the release of viral particles from infected cells, or gp19K, whose

constitutive expression reduces the host cytotoxic T cell response against the vector and increases the persistence of transgene expression on its own but possibly not in the context of constitutive expression of the entire E3 region.

The E3 region can be used to insert a second transgene, independent from the one inserted in the E1 region.

4. Limitations of the AdenoZAP Cloning System

The AdenoZAP cloning system will not be suitable when:

- the transgene contains both *Pac*I and *Swa*I sites
- the transgene contains all I-*Sce*I, *Sfi*I, *Alw*NI, *Bst*API, *Dra*III and *Pfl*MI sites.

If both *Pac*I and *Swa*I are present in the transgene, we suggest using the AdMax cloning system (Microbix), which does not necessitate plasmid linearization prior to transfection.

If I-*Sce*I, *Sfi*I, *Alw*NI, *Bst*API, *Dra*III and *Pfl*MI sites are all present in the transgene, we suggest the following alternatives:

- Partial digestion of your shuttle plasmid. Depending on the size of your transgene, no more than 600 ng left arm are necessary to set up the ligation with RightZAP, transfect and recover the virus.
- Generation of your recombinant virus by homologous DNA recombination in helper cells (e.g. 293 cells). Plasmids pZAP1.2 and pZAP2.2 are derivatives of, respectively, pZAP1.1 and pZAP2.1, in which a 404 bp-long Ad5 sequence was inserted immediately downstream from the multiple cloning site. This 400 bp sequence maps to nt 3504-3907 in the Ad5 genome and is identical to the first 400 bp of RightZAP1.1, 1.2, 2.1 and 2.2. To recover your recombinant virus, insert your expression cassette into the multiple cloning site of pZAP1.2 or pZAP2.2, linearize the resulting plasmid with *Pac*I or *Swa*I (whichever is not present in your expression cassette), and transfect the DNA into helper cells together with the right arm (RightZAP1.1 or RightZAP2.1 for WT E3; RightZAP1.2 or 2.2 for ΔE3). These vectors are sold separately. A detailed protocol for the method is provided with these new plasmids.

C. Safety Considerations About Adenovirus

1. Epidemiology

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

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

2. Clinical Manifestations

In adults, the most frequently reported illness has been acute respiratory disease caused by adenovirus type 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 type 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. Things to know about adenovirus biology and pertinent to your safety…

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

regions, in particular the E2 region promoter which controls viral genes involved in DNA replication. E1A-deleted adenoviruses are therefore considered as replication-deficient. Their replication requires the expression of the E1A products *in trans*, such as in 293 cells. However, some cell lines can express factors that functionally compensate for the loss of E1A expression in adenovirus infection and allow the virus to replicate. For instance, Hela cells, which express human papillomavirus (HPV) E6 and E7 regulatory proteins, whose functions are similar to those performed by Ad E1A and E1B 55-kDa proteins[Dyson, 1998 #137], support replication of E1-deleted adenoviruses when infected with high virus titers.[O'Connor, 2000 #136]

Ad5, the adenovirus strain at the origin of the vectors included in this kit, should pose no threat to humans. It has not been associated with malignancies in humans. However recent studies have shown that E1-subsituted adenovirus vectors can integrate into the cellular chromosomes *in vitro* with efficiencies ranging from ~10^{-3} to 10^{-5}.[Harui, 1999 #101] Although no such study has been conducted *in vivo*, extreme caution should be exercised, considering the high MOIs of adenovirus you will probably end up working with.

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

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

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

A BL2 laboratory should contain:

- A warning sign on the entrance door limiting the access to authorized persons only. The sign should identify the agent, list the name and

phone number of the lab director or other responsible person, and indicate any special requirement for entering the lab.

- A Class II biological safety cabinet. A Class II cabinet is a ventilated cabinet for personnel and product protection having an open front with inward airflow for personnel protection, and a HEPA filtered mass recirculated air flow for product protection. The face velocity of the inward flow of air through the full-width open front is 75 feet per minute or greater.
- At least one tissue culture incubator dedicated to infected cell cultures. Another separate incubator is desirable for growing uninfected cells.
- The minimal equipment to handle adenovirus culture without exiting the BL2 lab (such as centrifuges, microscope…).
- A sink for hand washing
- A chemical disinfectant kit or at least a gallon of bleach available for spills

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

- Always wear a lab coat while in the virus lab. Before exiting the laboratory for non-laboratory areas (e.g. cafeteria, library, administrative offices…), remove your lab coat and leave it in the laboratory.
- Avoid skin contamination with the virus. Always wear gloves (one pair OK, two pairs 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 bleach inside the hood before discarding them in the Biohazard trash and autoclaving. Place all materials to be decontaminated off-site in a durable leakproof container which is closed before removal. If possible, leave the contaminated materials in contact with bleach for a few hours before autoclaving (e.g. after rinsing your pipets with concentrated bleach inside the hood, soak them in a cylinder containing 10% bleach before autoclaving).
- Do not leave the BL2 laboratory with live viruses, unless they are in a sealed tube. Cell cultures transduced with adenoviruses should be inactivated either chemically or biochemically before leaving the BL2 facility.
- Store your adenovirus preparations at –70 °C in closed containers labeled with Biohazard warning signs.
- Wash your hands when exiting the laboratory.

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

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

7. More readings…

For further information about biosafety, we recommend reading the following publications from the NIH Division of Safety (http://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.

D. Protocols for Constructing Recombinant Adenoviruses using AdenoZAP1.x

1. Shuttle Plasmid Construction

The first step towards the construction of your recombinant adenovirus is the insertion of your expression cassette (promoter-cDNA-poly(A) signal) into the shuttle vector pZAP1.1. The purpose of this cloning is to link the expression cassette to the left adenovirus Inverted Terminal Repeat (ITR) and the packaging signal (Ad5 0-1 map unit).

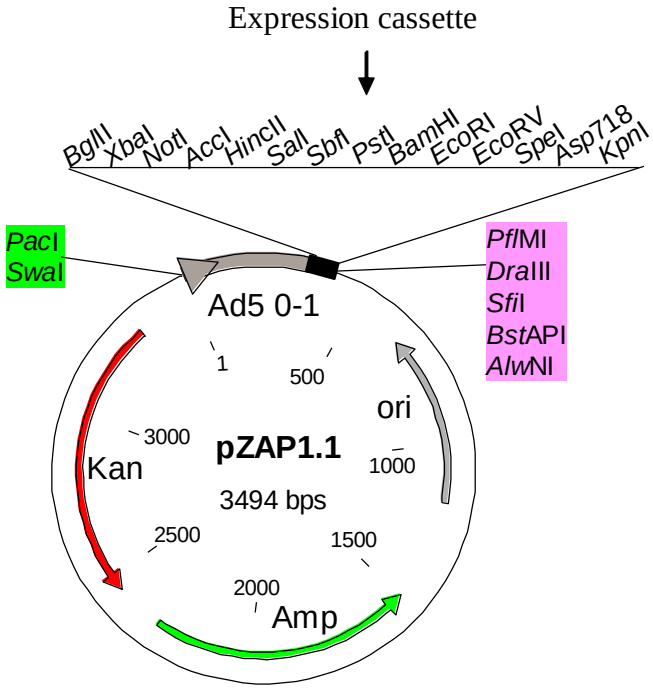


Fig. 3: pZAP1.1 Vector Map. The brown triangle and bar (Ad5 0-1) represent the adenovirus left ITR and packaging signal respectively. The black rectangle indicates the multiple cloning site. Kan: kanamycin-resistance gene (Tn 903). Amp: ampicillin-resistance gene. Ori: pUC19 origin of replication.

☺ Your expression cassette should contain a promoter and a poly(A) signal. 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 expression 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.

☺ You can select your recombinant clones using either ampicillin or kanamycin. If the plasmid from which you isolated your expression cassette contains an ampicillin-resistance gene, you will preferably select the recombinant clones on kanamycin-containing plates, and vice-versa.

☺ A small or medium-scale plasmid preparation (e.g. 50 mL LB) should yield enough DNA to perform the following steps. We have been able to rescue virus from mini-prep DNA obtained using the alkaline lysis method, although the yield of virus plaques was lower.

2. Excision of the "Left Arm" from the Shuttle Vector

- Digest 5 μg of shuttle vector containing your expression cassette with either *Pac*I or *Swa*I and with *Alw*NI, *Bst*API, *Sfi*I, *Dra*III or *Pfl*MI (whichever is not present in your expression cassette).

  ☺ Equimolar amounts of left and right arms are sufficient to complete an efficient ligation. The table below indicates the minimal amount of left arm necessary to set up one ligation reaction with 2 μg RightZAP1.1 (WT E3) or RightZAP1.2 (ΔE3), in function of the size of your insert. It will help you estimating how much shuttle plasmid DNA you will need to cut in order to obtain such amounts.

Table 2: Recommended Amount of Left Arm DNA Necessary to Set up one Ligation Reaction with 2 μg RightZAP. "Insert size" refers to the length of your expression cassette (including promoter-cDNA-poly(A) signal). Note that RightZAP1.1 does not accommodate inserts longer than 5.2 kb.

Insert Size (kb)	1	2	3	4	5	6	7	8
Left Arm DNA amount	100 ng	160 ng	230 ng	300 ng	360 ng	430 ng	500 ng	570 ng

- Purify the fragment containing your gene of interest on agarose gel.

  ☺ Silica-based methods for DNA fragment purification from agarose gels work well. We suggest QIAquick® (QIAGEN, Valencia, CA) for its rapidity (about 15 min).

- Verify the size and the integrity of the restriction fragment and estimate the DNA concentration on agarose gel.

3. Ligating the Left and Right Arms

1. Set up the following ligation reaction(s):

Table 3: Ligation Reaction between Left and Right Adenovirus Arms.

Reagent	Your Recombinant virus (Shuttle vector cut with *Pac*I)		Your Recombinant virus (Shuttle vector cut with *Swa*I)*		ΔE1 control virus (Optional)	
RightZAP1.1(or 1.2) (0.5μg/μl)	4 μl		4 μl		4 μl	
Left Arm	x μl		x μl		5 μl	(50 ng)
H_20	12-x μl		10-x μl		7 μl	
NaCl 1M			2 μl			
10x T4 DNA ligase buffer	2 μl		2 μl		2 μl	
*Pac*I or *Swa*I (10 units/μl)	1 μl	*Pac*I	1 μl	*Swa*I	1 μl	*Pac*I
T4 DNA ligase (400 units/μl)	1 μl		1 μl		1 μl	
Total volume	20 μl		20 μl		20 μl	

*This option is not available for AdenoZAP1.1 and 1.2 lots 0101.

☺ The addition of *Pac*I or *Swa*I in the ligation reaction is necessary to prevent the formation of DNA concatemers that would be unable to generate virus but would decrease the efficiency of virus recovery. If you used *Pac*I or *Swa*I to excise the left arm from the shuttle vector, add respectively *Pac*I or *Swa*I in the ligation reaction. Unlike *Pac*I, *Swa*I requires 100 mM NaCl for maximal activity. This does not inhibit the activity of the T4 DNA ligase.

☺ As explained in the Virus Recovery section (p. 23), you may duplicate the ligation reaction with the left arm containing your gene of interest in order to maximize your chances of recovering virus quickly.

☺ As a positive control, a 431-bp *Pac*I-*Dra*III fragment isolated from pZAP1.1 is provided. By ligating this fragment to RightZAP1.1 or 1.2, you will be able to construct an adenovirus containing a deletion in the E1 region but no expression cassette. This ΔE1 virus will be useful as negative control in your future experiments. Enough material is provided in the kit to perform this control ligation twice.

*Pac*I: New England Biolabs #R0547, 10 units/μl
*Swa*I: New England Biolabs #R0604, 10 units/μl
T4 DNA ligase: New England Biolabs #M0202S, 400 units/μl

- Incubate for 1 hour at room temperature.
- Inactivate the ligase by incubation at 65°C for 10 minutes.

☺ You may want to verify the ligation reaction before proceeding further. This can be done easily by running on agarose gel aliquots of the ligation reaction taken before addition of the ligase and after the reaction is stopped, and

observing band shifts. However, because of the large size of the right arm, band shifts are difficult to detect on an ordinary 0.7% agarose gel. Therefore we suggest digesting the aliquots with a restriction enzyme that does not cut inside the left arm and generates an adequate restriction pattern from the right arm. The text box below shows an example of such a digestion performed with *Bst*XI on the ligation product of RightZAP1.1 with the 431-bp ΔE1 control fragment.

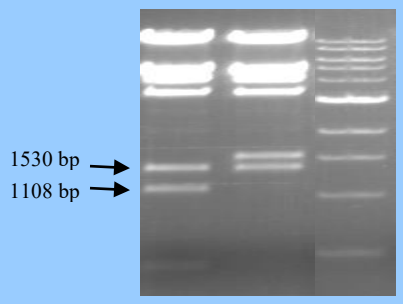
Checking the Ligation Reaction (Example)

- Set up the following reactions:

RightZAP2.1 (0.5 μg/μl)	4.4 μl	
Control ΔE1 Left Arm (10 ng/μl)	11.0 μl	
NaCl 1 M	2.2 μl	
H_2O	2.2 μl	
T4 DNA Ligase buffer (NEB)	2.2 μl	
Total volume	**22.0 μl**	
	10 μl	10μl
*Swa*I (10 u/μl)	-	0.5 μl
T4 DNA Ligase	-	0.5 μl

- Incubate for 1 hour at room temperature.
- Inactivate the T4 Ligase for 10 min. at 65°C.
- Add 0.5 μl *Bst*XI (10 u/μl; NEB # R0113S), incubate for 2 hours at 55°C.
- Load on a 0.7% agarose gel.

*Bst*XI generates a 1108-bp DNA fragment corresponding to the left end of RightZAP2.1. In presence of T4 DNA ligase, this fragment is almost completely shifted to form a 1530-bp DNA fragment with the 431-bp long ΔE1 control left arm.



- Precipitate the ligated DNA with ethanol:

  - To the ligation mixture (20 μl), add
    - 2 μl NaAc 3 M pH 5.2
    - 44 μl ethanol
  - Leave on ice for 10-15 min.
  - Centrifuge for 15 min. at maximum speed (14,000 rpm) in a minicentrifuge.

- Resuspend the DNA in 20 μl sterile TE pH 7.5.

☺ After centrifugation, remove the supernatant slowly in sterile conditions under the tissue culture hood. Spin again for a few seconds and take out the remaining supernatant. Let the pellet air-dry. When ready, add the TE buffer promptly. Do not dry your pellet too long and certainly not in a spin-vacuum, since large DNAs that have been dried too much are difficult to resuspend.

4. Virus Recovery

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

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

Because of the non-symmetrical nature of the sticky ends generated by excising the left arm from the shuttle vector, the ligation of the left and right arms should generate only one type of viral DNA molecule able to generate virus. Consequently, 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 4.

Briefly, two 293 cell dishes per construct are transfected with the DNA ligation mixture. The first dish is overlaid with agar. Three plaques are harvested, amplified and analyzed for transgene expression and genome stability. The

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

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.

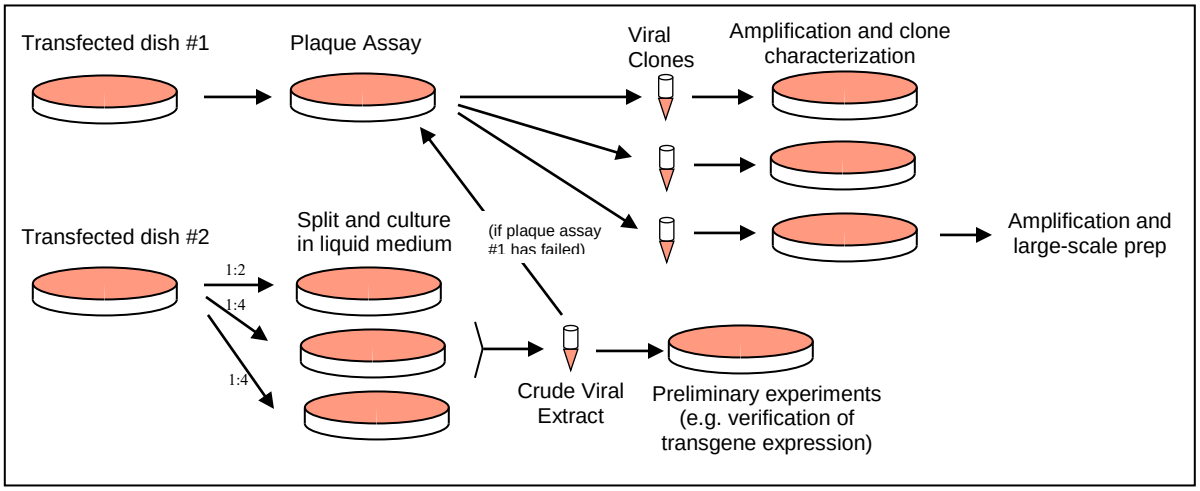


Figure 4: Proposed Flow-Chart to Isolate, Analyze and Amplify your Recombinant Virus.

a. Cell culture

- About one week before the expected transfection day, start culturing the 293 cells. Quickly thaw a vial of frozen 293 cells in a 37°C water bath and transfer the cells to a 10-cm dish containing 10 mL warm DMEM supplemented with 10% FCS 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, two positive controls: the positive control for the ligation reaction (which will generate a ΔE1 virus) and 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.

b. Transfection

☺ HEK 293 cells can be transfected very easily using the calcium-phosphate/DNA precipitation technique. The following recipe can yield up to 30 plaques/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
- 37.5 µL Na_2HPO_4 (or NaH_2PO_4) 1 M
- H_2O to 22 mL

Adjust to pH 7.0
Adjust volume to 25 mL with H2O
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 Falcon tube (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 cytophatic effect caused by the virus.

c. Virus Clone Isolation

One or two days after transfection, the cell monolayers should reach confluence.

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 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 fresh complete DMEM (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 (400g 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 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, 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.

Dish #2

- As soon as it becomes confluent, split the second transfected dish into three 6-cm dishes (1:4, 1:4, and 1:2)

  ☺ 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 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, and characterize your recombinant virus by restriction analysis.

- If the cell monolayers becomes 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. Feed the cells every 3-4 days.

E. Troubleshooting Guide

1. Cloning in pZAP Plasmid

Observation	Possible Cause(s)	Comments & Suggestions
The shuttle vector cannot be cut with a restriction enzyme in the multiple cloning site.	♦ Wrong buffer	♦ Use the buffer provided by the manufacturer. Mix the buffer thoroughly after thawing.
	♦ Wrong incubation temperature	♦ Verify the incubation temperature. In fact, all enzymes that cut in the multiple cloning site work at 37°C.
	♦ Enzyme is inactive.	♦ Test your enzyme with another DNA. Purchase new enzyme. ☺ All enzymes present in the multiple cloning site were tested successfully in the same pZAP vector batch that was sent to you!
Few or no colonies are obtained after transformation.	♦ Wrong antibiotic used, or too high concentration	♦ Use either 50μg/mL ampicillin, or 25 μg/mL kanamycin, or both.
	♦ Ligation reaction failed.	♦ Verify the integrity of your restriction fragments on agarose gel. ♦ Add a 3-fold molar excess of insert relative to the vector in the ligation reaction. ♦ Use fresh 10x T4 DNA ligase buffer, as the ATP it contains might be degraded. ♦ Use fresh T4 DNA ligase
	♦ Poor transformation efficiency	♦ Retransform, including a purified plasmid (e.g. pUC19) as positive control. Use another

		batch of competent cells. Transform by electroporation.
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/or 25 μg/mL kanamycin.
	♦ Contamination occurred with an amp^r or kan^r bacteria.	♦ Use Top10 (Invitrogen), DH5α or XL1-blue (Stratagene).
All clones lack the insert.	♦ Ligation did not work.	♦ Verify compatibility between the ends of your insert and the vector. ♦ Load an aliquot of your ligation mixture on gel and observe band shifts.
	♦ The linearized vector has compatible ends and recircularizes with itself.	♦ Treat the vector with a phosphatase (e.g. CIP) before purifying it on agarose gel. ♦ Add more insert relative to the vector in the ligation reaction (e.g. a 10-fold molar excess).
	♦ If you cut the vector with two enzymes in the multiple cloning site, one of them might have cut poorly or not at all and you are unable to detect it on agarose gel.	♦ Cut the vector with each enzyme separately, with its optimized buffer. ♦ Start cutting the vector with the enzyme you suspect working the least efficiently, and make sure its digestion is complete before cutting with the second enzyme. ♦ Design a 3-fragment ligation cloning strategy, using a third enzyme that cuts in a selectable marker (e.g. *Hin*dIII in the kan^r gene).
	♦ The digestion of the vector was not complete and the undigested form migrated together with the linear form on agarose	♦ Repeat the digest of the pZAP vector with less DNA and more enzyme. ♦ Purify the vector on a 0.7% agarose gel that is

	gel.	long enough to obtain a good separation between the linear and circular forms.
Insert is present but the restriction pattern is not the one expected.	♦ Your insert was inserted in the wrong orientation.	♦ Analyze more clones.
	♦ 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 – Stratagene)
The plasmid yield is poor.	♦ Not enough antibiotic added in the culture medium	♦ Make sure that you used either 50μg/mL ampicillin or 25 μg/mL kanamycin, make new antibiotic solutions if necessary.
	♦ Bacteria were cultured for a too long period of time before harvest.	♦ Harvest the bacteria when the culture reaches the end of the exponential growth phase (usually O.D.600 between 2.0 and 2.5).
	♦ Bacteria were not lyzed correctly during the purification.	♦ Follow carefully the recommendations of the manufacturer of your plasmid purification kit.

2. Excision of the "Left Arm" from the Shuttle Vector

Observation	Possible Cause(s)	Comments & Suggestions
The shuttle vector cannot be cut with a restriction enzyme at either side of the left arm.	♦ Wrong buffer	♦ Use the buffer provided by the manufacturer. Mix the buffer thoroughly after thawing.
	♦ Wrong incubation temperature	♦ Verify the incubation temperature. *Sfi*I and *Bst*API work best at 50 and 65 °C respectively. Perform these digestions in an oven rather than in a water bath in order to reduce H_2O evaporation and volume reduction.
	♦ Enzyme is inactive	♦ Test your enzyme with another DNA. Purchase new enzyme. ☺ All enzymes flanking the left arm sequences were tested successfully in the same pZAP vector batch that was sent to you!
The left arm cannot be separated from the vector backbone on agarose gel.	♦ The size of your expression cassette is about 2.7 kb	♦ Digest the shuttle plasmid containing your expression cassette with a restriction enzyme that cuts in the middle of the vector backbone, but not in the left arm (possibly *Hin*dIII, *Fsp*I, *Nru*I, *Xmn*I…).

3. Ligation of the "Left Arm" to the "Right Arm"

Observation	Possible Cause(s)	Comments & Suggestions
Ligation did not work (no difference is observed between the intensities of the left and right arms on agarose gel, before and after ligation).	♦ DNA ends are not compatible. One of the enzymes used to excise the left arm from the shuttle vector cuts inside your gene of interest.	♦ Visualize the "left arm" containing your gene of interest on agarose gel; verify its size and the absence of degradation. Use another enzyme or perform a partial digest.
	♦ DNA ends are degraded.	♦ Repurify the left arm using a high-quality plasmid prep and new reagents.
	♦ Not enough left arm was added in the ligation reaction.	♦ Estimate the concentration of the purified left arm on agarose gel by comparing its fluorescence intensity with that of a DNA ladder suitable for DNA quantification. Refer to Table 2 to estimate the minimal amount of DNA fragment that is needed for a complete ligation with 2 μg RightZAP.
	♦ Ligation buffer is expired. The ATP it contains may be degraded.	♦ Use fresh T4 DNA Ligase buffer.
	♦ T4 DNA ligase is inactive.	♦ Purchase new T4 DNA ligase (e.g. New England Biolabs #M0202S).
Ligation generated concatemers (as judged by comparing the samples on agarose gel, before and after ligation).	♦ *Pac*I or *Swa*I was omitted in the ligation reaction.	♦ If you excised the left arm from the shuttle vector using *Pac*I, add *Pac*I in the ligation reaction. Add *Swa*I if *Swa*I was used.

4. Virus Recovery

Observation	Possible Cause(s)	Comments & Suggestions
Do not get virus at all. No cytopathic effect is observed up to 12 days after transfection.	♦ The plasmid resulting from the insertion of your gene of interest into the shuttle vector pZAP is not correct.	♦ Verify the identity of the shuttle vector in which you inserted your gene of interest by restriction analysis. Verify that the enzymes you used to excise the left arm from the shuttle vector do not cut inside your gene of interest. Verify the presence of the left ITR/packaging signal sequences (a *Pac*I-*Bsr*GI digest should generate a 195 bp fragment).
	♦ The ligation of the left and right arms did not work.	♦ See p. 21 on how to verify the ligation efficiency.
	♦ The transfection was not efficient.	♦ Follow exactly the transfection protocol provided, paying 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-FCS about 8 hours after transfection.
	♦ Virus is likely to be unstable or difficult to construct.	♦ Check the length of your expression cassette. Remember that AdenoZAP1.1 and AdenoZAP2.1 do not accommodate inserts longer than 5.2 kb, while AdenoZAP1.2 and AdenoZAP2.2 do not accommodate inserts longer than 7.9 kb. If

		your expression cassette exceeds the transgene capacity of the virus, either no virus will appear or recombination will occur and generate a truncated mutant. ♦ 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. ♦ Monitor the transfection efficiency using the AdβGal DNA provided in the kit.
Do not get virus plaques in the dishes covered with agar, but well in those kept in liquid medium.	♦ Agar was too hot when poured and it killed the cells. In this case, the untransfected cells that were overlaid should have died too.	♦ Repeat the plaque assay using the crude viral extract obtained from the 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.	♦ Check the size of your insert: it may not exceed 5.2 kb for AdenoZAP1.1 and AdenoZAP2.1 or 7.9 kb for AdenoZAP1.2 and AdenoZAP2.2. ♦ Decrease the amount of "left arm" containing your gene of interest in the ligation reaction, and transfect helper cells again (the lesser copies of your supposedly toxic gene you transfect, the more likely the cells are going to survive) ♦ Use the AdenoQuick™ cloning system: systems that reconstitute the entire sequence of the recombinant adenovirus in a plasmid before transfection have been

		shown to produce more easily viruses that express toxic proteins.[Watzlik, 2000 #106] ♦ Use an inducible expression system.
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.
	♦ Virus is unstable.	♦ Analyze transgene expression with other virus clones.
	♦ 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.

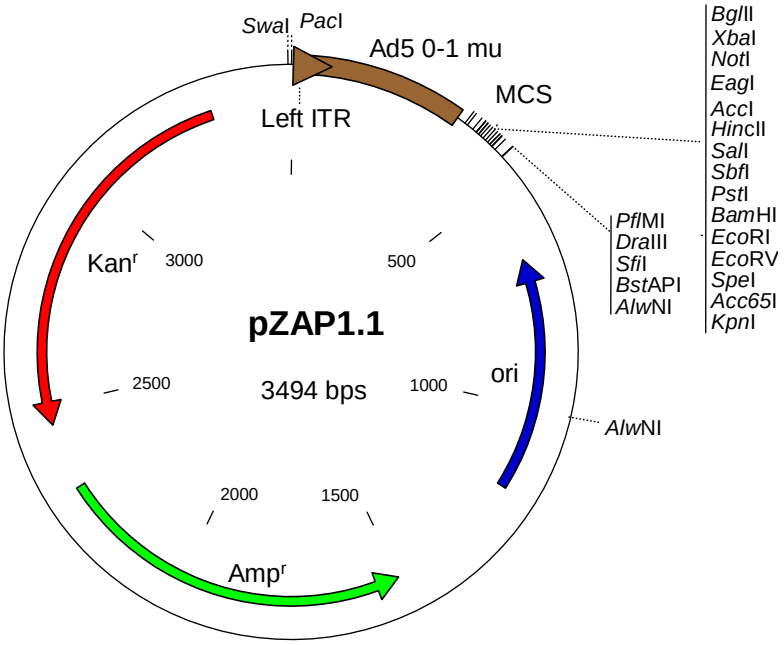
F. Plasmid Maps

pZAP1.1

3,494 base pairs
Sequence available at www.od260.com

pZAP1.1 is a 3.5 kb shuttle vector designed for inserting expression cassettes in place of the E1 region of the Ad5 genome, in combination with AdenoZAP1.x system. It contains a multiple cloning site (MCS) downstream from the first map unit (mu) of the Ad5 genome. Expression cassettes inserted into this site should contain promoter, cDNA, and polyA signal. The left arm DNA (which corresponds to the left ITR, packaging signal and expression cassette) can be excised from the vector with either *Pac*I or *Swa*I on one side, and either *Pfl*MI, *Dra*III, *Sfi*I, *Bst*API or *Alw*NI on the other side.

Feature	Coordinates	Source
left ITR	5-107	Ad5
0-1 mu	5-357	Ad5
origin of replication	1238-650	pUC19
Amp^r	2269-1409	*Tn3*
Kan^r	3261-2446	*Tn903*



pZAP1.1 MCS

```
                                                    HindII     BamHI
                                  (SmaI)[1]  EagI     AccI|     PstI|
Ad5 0-1 mu          BglII  XbaI(AvaI) |  NotI    SalI||    SbfI| EcoRI
                      |      |      | |     |       |||       ||     |
          GTCTAGGGAGATCTTCTAGACCCGGGAGCGGCCGCTGTCGACCTGCAGGATCCGAATTCG
      350 +---------+---------+---------+---------+---------+---------+--------- 409
          CAGATCCCTCTAGAAGATCTGGGCCCTCGCCGGCGACAGCTGGACGTCCTAGGCTTAAGC

            SpeI      KpnI DraIII                        AlwNI
 EcoRV     |Acc65I     |   PflMI       SfiI              BstAPI
    |      |     |     |     |          |                   |
          ATATCACTAGTGGTACCCACGAGGTGGCCAGAGAGGCCGAGGTGGCAGGAGCTGCATCGA
      410 +---------+---------+---------+---------+---------+---------+--------- 469
          TATAGTGATCACCATGGGTGCTCCACCGGTCTCTCCGGCTCCACCGTCCTCGACGTAGCT
```

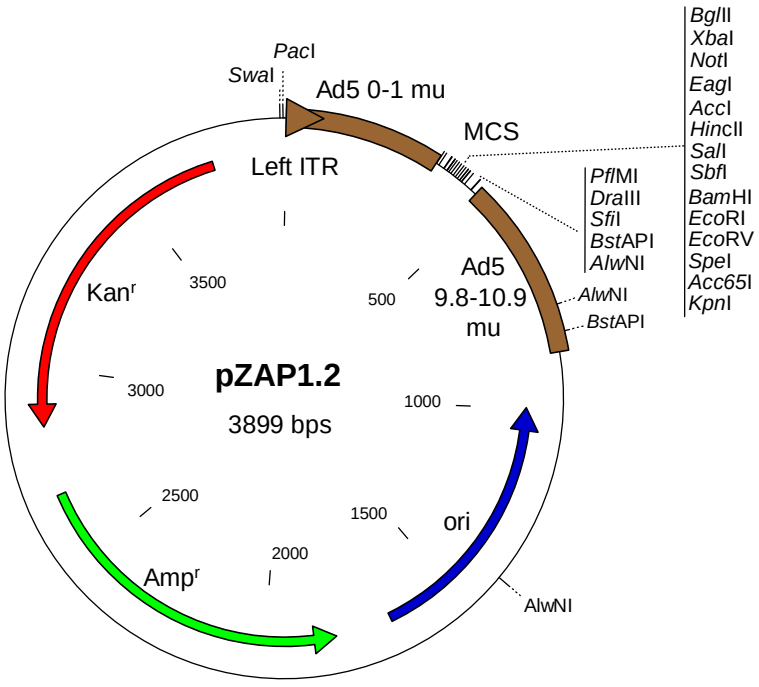
[1] Restriction sites between brackets are not unique.

pZAP1.2

3,899 base pairs
Sequence available at www.od260.com

pZAP1.2 is a shuttle vector designed for inserting expression cassettes in place of the E1 region of the Ad5 genome, in combination with AdenoZAP1.x system. In contrast to pZAP1.1 it can be used either in the *in vitro* DNA ligation approach or in the approach based on homologous DNA recombination in helper cells. It contains a multiple cloning site located between the first map unit (mu) of the Ad5 genome and a 404 bp-long Ad5 sequence corresponding to mu 9.8-10.9 (psn 3504-3907) in the Ad5 genome. Expression cassettes inserted into this site should contain a promoter, cDNA, and polyA signal. When the *in vitro* DNA ligation approach is used, the left arm DNA (which corresponds to the left ITR, packaging signal and expression cassette) can be excised from the vector with either *Pac*I or *Swa*I on one side, and either *Pfl*MI, *Dra*III, *Sfi*I, *Bst*API or *Alw*NI on the other side. When recombination in helper cells is used, the shuttle vector needs to be linearized using *Pac*I or *Swa*I only.

Feature	Coordinates	Source
left ITR	5-107	Ad5
0-1 mu	5-357	Ad5
9.8-10.9 mu	468-871	Ad5
origin of replication	1643-1055	pUC19
Amp^r	2674-1814	*Tn3*
Kan^r	3666-2851	*Tn903*



pZAP1.2 MCS

```
                                        HindII
                          (SmaI)[2] EagI     AccI|    BamHI
Ad5 0-1 mu     BglII  XbaI(AvaI) |  NotI    SalI||    SbfI| EcoRI   EcoRV
               |      |      | |  |       |   |||       ||     |       |
          TAGGGAGATCTTCTAGACCCGGGAGCGGCCGCTGTCGACCTGCAGGATCCGAATTCGATA
      353 -------+---------+---------+---------+---------+---------+-- 412
          ATCCCTCTAGAAGATCTGGGCCCTCGCCGGCGACAGCTGGACGTCCTAGGCTTAAGCTAT

             Acc65I     DraIII                      AlwNI
          SpeI     |KpnI  PflMI      SfiI           BstAPI          Ad5 9.8-10.5 mu
            |      |   |      |        |                |          |
          TCACTAGTGGTACCCACGAGGTGGCCAGAGAGGCCGAGGTGGCAGGAGCTGCATCGATTG
      413 -------+---------+---------+---------+---------+---------+-- 472
          AGTGATCACCATGGGTGCTCCACCGGTCTCTCCGGCTCCACCGTCCTCGACGTAGCTAAC
```

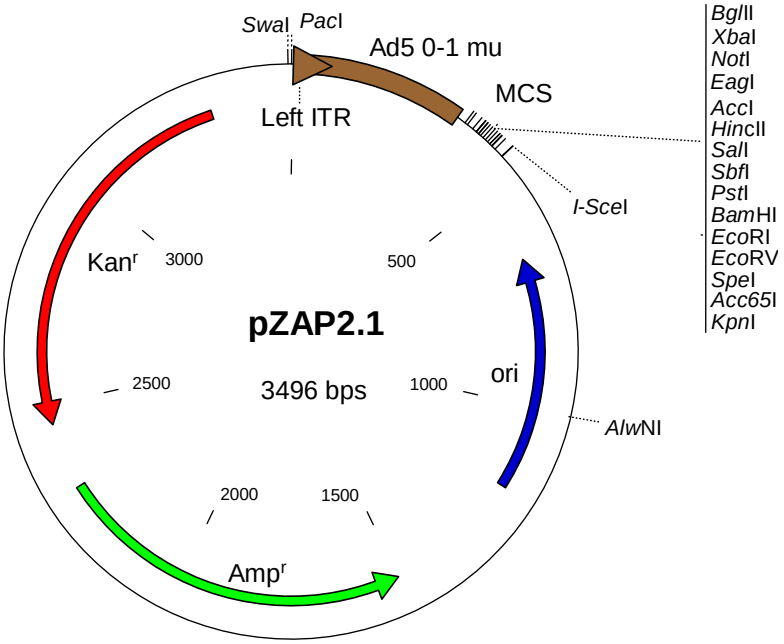
[2] Restriction sites between brackets are not unique.

pZAP2.1

3,496 base pairs
Sequence available at www.od260.com

pZAP2.1 is a shuttle vector designed for inserting expression cassettes in place of the E1 region of the Ad5 genome, in combination with AdenoZAP2.x system. It contains a multiple cloning site (MCS) downstream from the first map unit (mu) of the Ad5 genome. Expression cassettes inserted into this site should contain a promoter, cDNA, and polyA signal. The left arm DNA (which corresponds to the left ITR, packaging signal and expression cassette) can be excised from the vector with either *Pac*I or *Swa*I on one side, and *I-Sce*I on the other side.

Feature	Coordinates	Source
left ITR	5-107	Ad5
0-1 mu	5-357	Ad5
origin of replication	1238-650	pUC19
Amp^r	2269-1409	*Tn3*
Kan^r	3261-2446	*Tn903*



pZAP2.1 MCS

```
                                                  HindII    BamHI
                                    (SmaI)[3] EagI    AccI|    PstI|
Ad5 0-1 mu          BglII  XbaI(AvaI) |  NotI    SalI||    SbfI| EcoRI
                    |      |      | |      |       |||       ||      |
          GTCTAGGGAGATCTTCTAGACCCGGGAGCGGCCGCTGTCGACCTGCAGGATCCGAATTCG
      350 +---------+---------+---------+---------+---------+---------+--------- 409
          CAGATCCCTCTAGAAGATCTGGGCCCTCGCCGGCGACAGCTGGACGTCCTAGGCTTAAGC

            SpeI      KpnI        (SmaI)
       EcoRV   |Acc65I   |      (AvaI) |          I-SceI
          |    |    |    |           | |           |
          ATATCACTAGTGGTACCCACCGCGCCCCCGGGATTACCCTGTTATCCCTAGAGCTGCATC
      410 +---------+---------+---------+---------+---------+--------- 469
          TATAGTGATCACCATGGGTGGCGCGGGGGCCCTAATGGGACAATAGGGATCTCGACGTAG
```

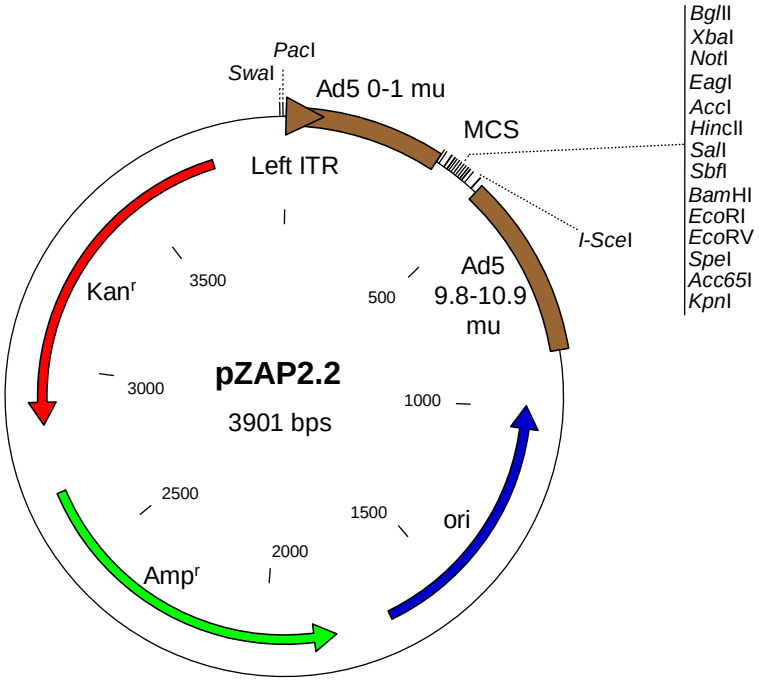
[3] Restriction sites between brackets are not unique.
The recognition site for I-SceI is highlighted in yellow.

pZAP2.2

3,901 base pairs
Sequence available at www.od260.com

pZAP2.2 is a shuttle vector designed for inserting expression cassettes in place of the E1 region of the Ad5 genome, in combination with AdenoZAP2.x system. In contrast to pZAP2.1 it can be used either in the *in vitro* DNA ligation approach or in the approach based on homologous DNA recombination in helper cells. It contains a multiple cloning site located between the first map unit (mu) of the Ad5 genome and a 404 bp-long Ad5 sequence corresponding to mu 9.8-10.9 (psn 3504-3907) in the Ad5 genome. Expression cassettes inserted into this site should contain a promoter, cDNA, and polyA signal. In case the *in vitro* DNA ligation approach is used, the left arm DNA (which corresponds to the left ITR, packaging signal and expression cassette) can be excised from the vector with either *Pac*I or *Swa*I on one side, and *I-Sce*I on the other side. In case the recombination in helper cells is used, the shuttle vector needs to be linearized using *Pac*I or *Swa*I only.

Feature	Coordinates	Source
left ITR	5-107	Ad5
0-1 mu	5-357	Ad5
9.8-10.9 mu	470-873	Ad5
origin of replication	1645-1057	pUC19
Amp^r	2676-1816	*Tn3*
Kan^r	3668-2853	*Tn903*



pZAP2.2 MCS

```
                                                  HindII
                          (SmaI)[4]  EagI     AccI|     BamHI
Ad5 0-1 mu     BglII  XbaI(AvaI) |  NotI     SalI||     SbfI| EcoRI    EcoRV
                  |      |      | |      |        |||        ||      |        |
           TAGGGAGATCTTCTAGACCCGGGAGCGGCCGCTGTCGACCTGCAGGATCCGAATTCGATA
       353 -------+---------+---------+---------+---------+---------+---------+-- 412
           ATCCCTCTAGAAGATCTGGGCCCTCGCCGGCGACAGCTGGACGTCCTAGGCTTAAGCTAT

              Acc65I
         SpeI     |KpnI                          I-SceI                    Ad5 9.8-10.5 mu
           |      |   |                             |                        |
           TCACTAGTGGTACCCACCGCGCCCCCGGGATTACCCTGTTATCCCTAGAGCTGCATCGAT
       413 -------+---------+---------+---------+---------+---------+---------+-- 472
           AGTGATCACCATGGGTGGCGCGGGGGCCCTAATGGGACAATAGGGATCTCGACGTAGCTA
```

[4] Restriction sites between brackets are not unique. The recognition site for I-SceI is highlighted in yellow.

G.List of Components and Other Useful Information

AdenoZAP1.1 Kit Cat # ZK-001

The AdenoZAP1.1 Kit contains the components necessary to construct Ad5-based adenovirus vectors containing transgenes in place of the E1 region and characterized by a wild-type E3 region (Fig.1). The cloning strategy is based on the directional ligation of restriction fragments obtained using *Sfi*I-like restriction enzymes. The cloning capacity is 5.2 kb. The kit contains enough materials to construct 10 recombinant viruses.
In addition to the shuttle vector pZAP1.1 and the right arm RightZAP1.1, two controls are provided:

1. a ΔE1 left arm, which can be used as a control for the ligation reaction with RightZAP1.1 and will generate a ΔE1-adenovirus that can be used as a negative control in future experiments,
2. a plasmid-based adenovirus DNA (Adßgal), which can be used as a control to monitor the transfection efficiency and will generate an adenovirus expressing a ß-galactosidase under the control of a CMV promoter.

Contents:	**Cat. #**	
• pZap1.1	ZP-01	20 μg
• RightZAP1.1 (WT E3)	ZR-01	20 μg (good for 10 virus constructions)
• Control ΔE1left Arm	ZL-01	100 ng (good for 2 control ligations)
• Control Adβgal DNA	ZC-01	10 μg (good for 5 transfections)
• Short Manual	ZM-01	

AdenoZAP1.2 Kit Cat # ZK-003

The AdenoZAP1.2 Kit contains the components necessary to construct Ad5-based adenovirus vectors containing transgenes in place of the E1 region and characterized by a 2.7 kb deletion in the E3 region (Fig.1). The cloning strategy is based on the directional ligation of restriction fragments obtained using *Sfi*I-like restriction enzymes. The cloning capacity is 7.9 kb. The kit contains enough materials to construct 10 recombinant viruses.
In addition to the shuttle vector pZAP1.1 and the right arm RightZAP1.2, two controls are provided:

1. a ΔE1 left arm, which can be used as a control for the ligation reaction with RightZAP1.2 and will generate a ΔE1-adenovirus that can be used as a negative control in future experiments,
2. a plasmid-based adenovirus DNA (Adßgal), which can be used as a control to monitor the transfection efficiency and will generate an adenovirus expressing a ß-galactosidase under the control of a CMV promoter.

Contents:	**Cat. #**	
• pZap1.1	ZP-01	20 μg
• RightZAP1.2 (ΔE3)	ZR-02	20 μg (good for 10 constructions)
• Control ΔE1left Arm	ZL-01	100 ng (good for 2 control ligations)
• Control Adβgal DNA	ZC-01	10 μg (good for 5 transfections)
• Short Manual	ZM-01	

AdenoZAP2.1 Kit Cat # ZK-009

The AdenoZAP2.1 Kit contains the components necessary to construct Ad5-based adenovirus vectors containing transgenes in place of the E1 region and characterized by a wild-type E3 region (Fig.2). The cloning strategy is based on the directional ligation of restriction fragments obtained using the I-*Sce*I endonuclease. The cloning capacity is 5.2 kb. The kit contains enough materials to construct 10 recombinant viruses.
In addition to the shuttle vector pZAP2.1 and the right arm RightZAP2.1, two controls are provided:

1. a ΔE1 left arm, which can be used as a control for the ligation reaction with RightZAP2.1 and will generate a ΔE1-adenovirus that can be used as a negative control in future experiments,
2. a plasmid-based adenovirus DNA (Adßgal), which can be used as a control to monitor the transfection efficiency and will generate an adenovirus expressing a ß-galactosidase under the control of a CMV promoter.

Contents:	**Cat. #**	
• pZap2.1	ZP-02	20 μg
• RightZAP2.1 (WT E3)	ZR-03	20 μg (good for 10 virus constructions)
• Control ΔE1left Arm	ZL-02	100 ng (good for 2 control ligations)
• Control Adβgal DNA	ZC-01	10 μg (good for 5 transfections)
• Short Manual	ZM-02	

AdenoZAP2.2 Kit Cat # ZK-010

The AdenoZAP2.2 Kit contains the components necessary to construct Ad5-based adenovirus vectors containing transgenes in place of the E1 region and characterized by a 2.7 kb deletion in the E3 region (Fig.2). The cloning strategy is based on the directional ligation of restriction fragments obtained using the I-*Sce*I endonuclease. The cloning capacity is 7.9 kb. The kit contains enough materials to construct 10 recombinant viruses.
In addition to the shuttle vector pZAP2.1 and the right arm RightZAP2.2, two controls are provided:

1. a ΔE1 left arm, which can be used as a control for the ligation reaction with RightZAP2.2 and will generate a ΔE1-adenovirus that can be used as a negative control in future experiments,
2. a plasmid-based adenovirus DNA (Adßgal), which can be used as a control to monitor the transfection efficiency and will generate an adenovirus expressing a ß-galactosidase under the control of a CMV promoter.

Contents:	**Cat. #**	
• pZap2.1	ZP-02	20 μg
• RightZAP2.2 (ΔE3)	ZR-04	20 μg (good for 10 constructions)
• Control ΔE1left Arm	ZL-02	100 ng (good for 2 control ligations)
• Control Adβgal DNA	ZC-01	10 μg (good for 5 transfections)
• Short Manual	ZM-02	

RightZAP1.1 DNA Cat # ZR-01

The RightZAP1.1 DNA is a 32.5 kb linear DNA fragment that encompasses bp 3504-right end of the Ad5 genome (Fig.1). The E3 region is intact. The DNA was purified from a cosmid. It is used in combination with the shuttle vector pZAP1.1 to construct replication-deficient adenoviruses containing transgenes in place of the E1 region of the Ad5 genome. The maximum transgene capacity of the resulting virus is 5.2 kb.

Contents:	**Cat. #**	
• RightZAP1.1 (WT E3)	ZR-01	20 μg (good for 10 virus constructions)

RightZAP1.2 DNA Cat # ZR-02

The RightZAP1.2 DNA is a 29.8 kb linear DNA fragment that encompasses bp 3504-right end of the Ad5 genome (Fig.1). The E3 region is deleted (2.7 kb). The DNA was purified from a cosmid. It is used in combination with the shuttle vector pZAP1.1 to construct replication-deficient adenoviruses containing transgenes in place of the E1 region of the Ad5 genome. The maximum transgene capacity of the resulting virus is 7.9 kb.

Contents:	**Cat. #**	
• RightZAP1.2 (ΔE3)	ZR-02	20 μg (good for 10 virus constructions)

RightZAP2.1 DNA Cat # ZR-03

The RightZAP2.1 DNA is a 32.5 kb linear DNA fragment that encompasses bp 3504-right end of the Ad5 genome (Fig.2). The E3 region is intact. The DNA was purified from a cosmid. It is used in combination with the shuttle vector pZAP2.1 to construct replication-deficient adenoviruses containing transgenes in place of the E1 region of the Ad5 genome. The maximum transgene capacity of the resulting virus is 5.2 kb.

Contents:	**Cat. #**	
• RightZAP2.1 (WT E3)	ZR-03	20 μg (good for 10 virus constructions)

RightZAP2.2 DNA Cat # ZR-04

The RightZAP2.2 DNA is a 29.8 kb linear DNA fragment that encompasses bp 3504-right end of the Ad5 genome (Fig.2). The E3 region is deleted (2.7 kb). The DNA was purified from a cosmid. It is used in combination with the shuttle vector pZAP2.1 to construct replication-deficient adenoviruses containing transgenes in place of the E1 region of the Ad5 genome. The maximum transgene capacity of the resulting virus is 7.9 kb.

Contents:	**Cat. #**	
• RightZAP2.2 (Δ E3)	ZR-04	20 μg (good for 10 virus constructions)

pZAP1.1 Cat # ZP-01

pZAP1.1 is a 3.5 kb shuttle vector designed for inserting expression cassettes in place of the E1 region of the Ad5 genome, in combination with the AdenoZAP1.x system (Fig.1). It contains a multiple cloning site downstream from the first map unit of the Ad5 genome. Expression cassettes inserted into this site should contain promoter, cDNA, and polyA signal. The left arm DNA (which corresponds to the left ITR, packaging signal and expression cassette) can be excised from the vector with either *Pac*I or *Swa*I on the left side, and either *Pfl*MI, *Dra*III, *Sfi*I, *Bst*API or *Alw*NI on the right side.

Contents:	**Cat. #**	
• pZAP1.1	ZP-01	20 μg

pZAP2.1 Cat # ZP-02

pZAP2.1 is a 3.5 kb shuttle vector designed for inserting expression cassettes in place of the E1 region of the Ad5 genome, in combination with the AdenoZAP2.x system (Fig.2). It contains a multiple cloning site downstream from the first map unit of the Ad5 genome. Expression cassettes inserted into this site should contain promoter, cDNA, and polyA signal. The left arm DNA (which corresponds to the left ITR, packaging signal and expression cassette) can be excised from the vector with either *Pac*I or *Swa*I on the left side, and I-*Sce*I on the right side.

Contents:	**Cat. #**	
• pZAP2.1	ZP-02	20 μg

Control ΔE1 Left Arm (Sfi) Cat # ZL-01

The Control ΔE1 Left Arm (Sfi) is a 431 bp-long *Pac*I-*Dra*III DNA fragment isolated from pZAP1.1. It contains the left ITR, packaging signal and the multiple cloning site. This DNA fragment can be used to control the efficiency of the ligation with RightZAP1.1 and RightZAP1.2. By transfecting the ligation mixture into helper cells (e.g. 293 cells), you will be able to construct an E1-deleted adenovirus without expression cassette, which will be useful as negative control in your future experiments. Enough material is provided to perform this control ligation twice.

Contents:	**Cat. #**	
• Control ΔE1 Left Arm (Sfi)	ZL-01	100 ng

Control ΔE1 Left Arm (Sce) Cat # ZL-02

The Control ΔE1 Left Arm (Sce) is a 453 bp-long *Swa*I-*Sce*I DNA fragment isolated from pZAP2.1. It contains the left ITR, packaging signal and the multiple cloning site. This DNA fragment can be used to control the efficiency of the ligation with RightZAP2.1 and RightZAP2.2. By transfecting the ligation mixture into helper cells (e.g. 293 cells), you will be able to construct an E1-deleted adenovirus without expression cassette, which will be useful as negative control in your future experiments. Enough material is provided to perform this control ligation twice.

Contents:	**Cat. #**	
• Control ΔE1 Left Arm (Sce)	ZL-02	100 ng

Control Adßgal DNA Cat # ZC-01

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

Contents:	**Cat. #**	
• Control Adßgal DNA	ZC-01	10 µg

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