

**Precautions for the use of this product**

- Please follow the guideline for experiments using recombinant DNA issued by the relevant authorities and the safety committee of your organization or your country in using this product.
- The use of this product is limited for research purposes. It must not be used for clinical purposes or for *in vitro* diagnosis.
- Individual license agreement must be concluded when this product is used for industrial purposes.
- In this kit, recombinant viral particles infectious to mammalian cells are produced in 293 cells. Although this recombinant virus cannot proliferate in cells other than 293 cells, in case that it attaches to the skin or the airway, it efficiently enters cells and expresses the target gene. Please use a safety cabinet to prevent inhalation or adhesion of the virus.
- Basic techniques of genetic engineering and cell cultivation are needed for the use of this product.
- The user is strongly advised not to generate recombinant adenovirus capable of expressing known oncogenes and any genes known to be hazardous to the mammals.
- Takara is not liable for any accident or damage caused by the use of this product.

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## I. Introduction

### I-1. Adenovirus vectors

Adenovirus vectors are widely used as versatile expression vectors in various areas ranging from basic research to actual applications including functional analysis in animals as well as in cultured cells, because of their high utility and the other advantages listed below.

- (1) Useful for research about function of a gene of interest at a particular stage, as it highly and transiently express the gene.
- (2) It can be used for efficient transduction in a wide species of mammalian cells of not only human cells but mouse and rat cells. In addition, it can infect in resting cells, proliferating cells, undifferentiated cultured cells, and differentiated cells including nervous system cells.
- (3) A high-titer virus can be obtained.  
Virus solution of  $10^8 \sim 10^9$  pfu/ml can be obtained with ease. They can further be concentrated to about  $10^{11}$  pfu/ml.
- (4) It can be used for efficient transduction of a gene by direct administration to an animal body.

The adenovirus vectors most commonly used today are produced from human adenovirus serotype 5 (Ad5) with the E1 and E3 genes deleted. The E1 deletion renders these recombinant adenoviruses defective for replication in normal cells; they can only replicate in 293 cells (human embryonic kidney derived established cell line)<sup>1)</sup>, which express the E1 gene. The E3 gene is not essential for viral replication *in vitro*; it is known to be associated with immune surveillance *in vivo*.

The Adenovirus Expression Vector Kit (Dual Version) allows production of standard Ad5-derived recombinant adenoviruses with E1 and E3 deletions. These recombinant adenoviruses are defective for replication and can replicate only in 293 cells since they lack the E1 gene. These adenovirus vectors allow for the insertion of up to 7 kbp of foreign DNA.

### I-2. Product overview

Adenovirus Expression Vector Kit (Dual Version) Ver.2 is designed for making recombinant adenovirus producing by either of two methods; the full-length DNA transfer method or the COS-TPC method<sup>2)</sup>.

Dr. Saito *et al.* at the Institute of Medical Science at the University of Tokyo developed the full-length DNA transfer method<sup>4)</sup> for simple production of recombinant adenovirus by constructing a dual cosmid<sup>3)</sup> containing adenovirus DNA lacked E1 and E3 genes, which is a modified version of the cosmid vector for the COS-TPC method. The full-length DNA transfer method is for producing a target recombinant adenovirus by transduction only the plasmid containing full-length adenovirus into 293 cells.

This product contains the dual cosmid having *Bsp*T140 I and *Pac* I restriction sites outside of both terminal ends of virus genome. After a gene of interest is inserted in the dual cosmid, the recombinant adenovirus having the gene of interest is able to be obtained by transfection into 293 cells with the *Bsp*T140 I or *Pac* I-digested recombinant cosmid. In the kit Ver.2, you can select the restriction enzyme, *Bsp*T140 I or *Pac* I, which will be used for cutting the recombinant cosmid. Therefore, it is possible to produce the recombinant adenovirus having your gene of interest by the full-length DNA transfer method.

In a difficult case for production of a recombinant adenovirus using the full-length DNA transfer method based on sequence or function of a gene of interest, the COS-TPC method is available for production of recombinant adenovirus by co-transfection

of the obtained recombinant cosmid together with Adenovirus genome DNA-TPC (Cat.#6171) into 293 cells. There is no difference in the structure or characteristics of recombinant viruses produced by both methods.

## II. General Information

Recombinant adenoviruses are produced by the outline below. See section VII. Procedure for detailed protocol.

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### Outline of the generation of recombinant adenovirus

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#### A. Preparation of the recombinant cosmid.

↓ Insertion of the target gene into the cosmid vector, and prepare the recombinant cosmid.

#### B. Generation of recombinant adenovirus.

↓ Transfect the recombinant cosmid prepared in A. into 293 cells and generate the recombinant adenovirus.

#### C. Preparation of high-titer recombinant adenovirus.

Prepare large-scale recombinant adenovirus with a viral titer of  $10^9$  PFU/ml.

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### <1> Selection of cosmid vectors

Select a cosmid vector based on the characteristics of the insert DNA and the purpose of the experiment prior to the start of recombinant cosmid preparation. See section IV-2. Cosmid vector structures for detailed information on cosmid vectors.

- **pAxCawtit2** : suitable for high-level expression in a broad range of cell types  
The pAxCawtit vector allows for high-level expression in broad range of cell types driven by the CAG promoter, a strong mammalian promoter<sup>9)</sup>, and accommodates inserts of up to 5 kbp of foreign DNA.
- **pAxcwit2** : suitable for use with the a promoter of the user's choice  
The pAxcwit vector does not contain a promoter and polyA signal, and accommodates expression cassettes (chosen promoter + target gene + poly (A) signal) of up to 7 kbp.

**Note** : Both vectors pAxcwit2 and pAxCawtit2 are included in this kit.

### <2> Selection of recombinant adenovirus preparation method

Select either the full-length DNA transfer method or the COS-TPC method prior to the start of recombinant adenovirus preparation.

The full-length DNA transfer method is generally sufficient for recombinant virus preparation, but if efficiency of virus preparation is low and viruses are difficult to obtain with the method because of various factors such as those associated with the characteristics of the gene of interest, the COS-TPC method may allow for more efficient recombinant virus preparation (see section VIII. Q&A. Q1). See section III. Principles of recombinant adenovirus preparation for detailed principles of the two methods.

- First choice → full-length DNA transfer method
- For more efficient virus preparation → COS-TPC method
- When an insert DNA contains the *Bsp*T104 I (TTCGAA) and *Pac* I (TTAATTAA) recognition site → COS-TPC method

**Note :** The COS-TPC method requires Adenovirus genome DNA-TPC (Cat.#6171).

### III. Principles of recombinant adenovirus preparation

#### III-1. Full-length DNA transfer method

The full-length DNA transfer method is a recombinant adenovirus preparation method to transfect recombinant cosmids (i.e., cosmid vectors with target gene) digested with restriction enzymes into 293 cells (Figure 1-1).

The dual cosmids supplied with the Adenovirus Expression Vector Kit (Dual Version) contain the complete adenovirus genome, except for the E1 and E3 genes, and *Bsp*T104 I (*Csp*45 I) and *Pac* I sites flanking the adenovirus genome. This design allows for preparation of recombinant adenoviruses by simply digesting with *Bsp*T104I or *Pac* I the recombinant cosmids created by inserting the target gene into the cosmid vectors and transfecting them into 293 cells.

This method eliminates the chance of parent virus contamination and produces a high yield of the desired virus since it does not require homologous recombination within cells. However, the characteristics of the target gene and other factors may reduce the efficiency of virus preparation in some cases (See VIII. Q&A. Q1).

#### III-2. COS-TPC method

The COS-TPC method is a recombinant adenovirus preparation method to co-transfect recombinant cosmids and Adenovirus genome DNA-TPC (Cat.#6171; Optional) into 293 cells by means of homologous recombination (Figure 1-2).

The Adenovirus genome DNA-TPC contains terminal proteins (TPs), which are bound to the ends of adenovirus genome DNA, enabling more efficient and reliable recombinant adenovirus preparation than the full-length DNA transfer method.

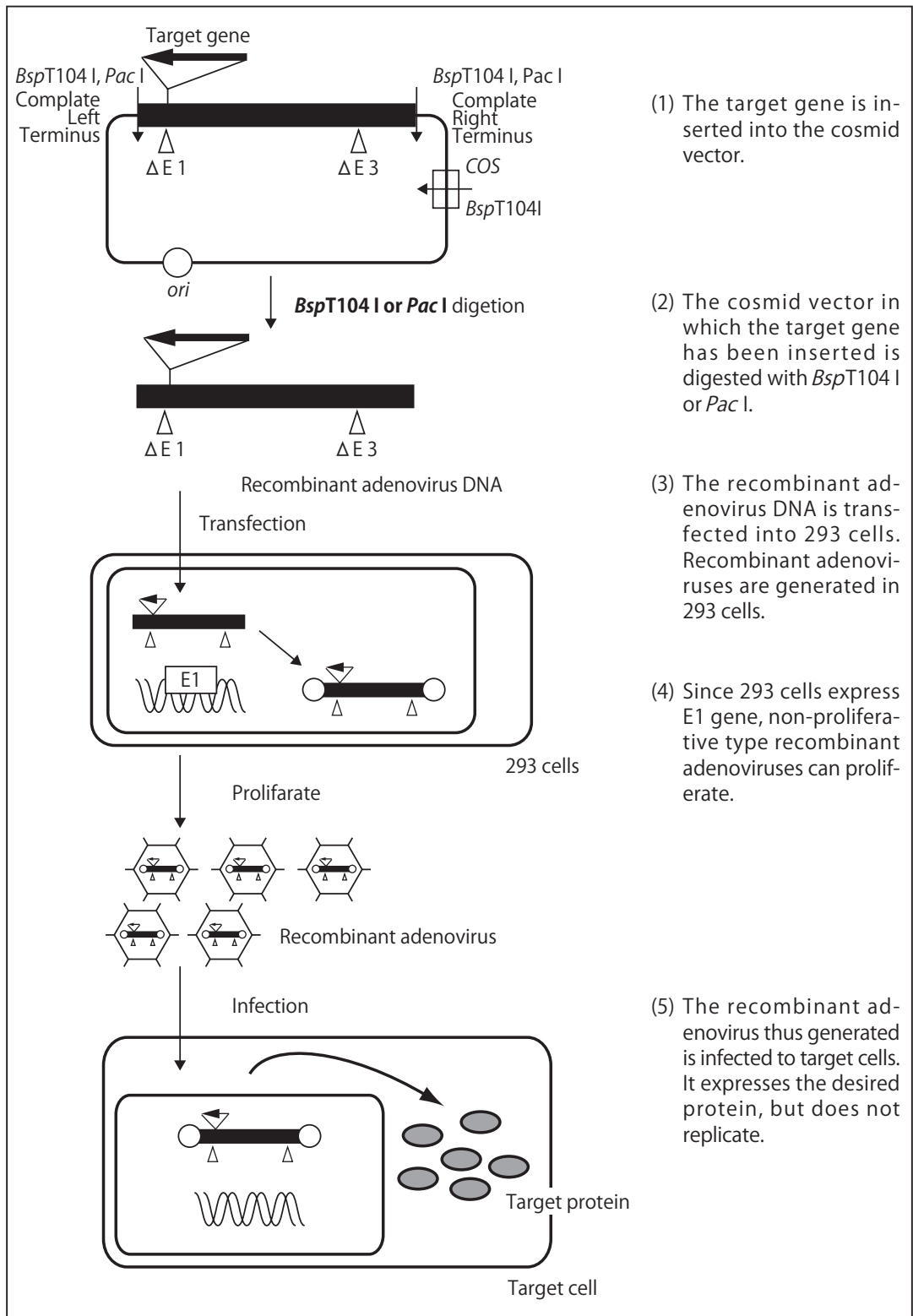
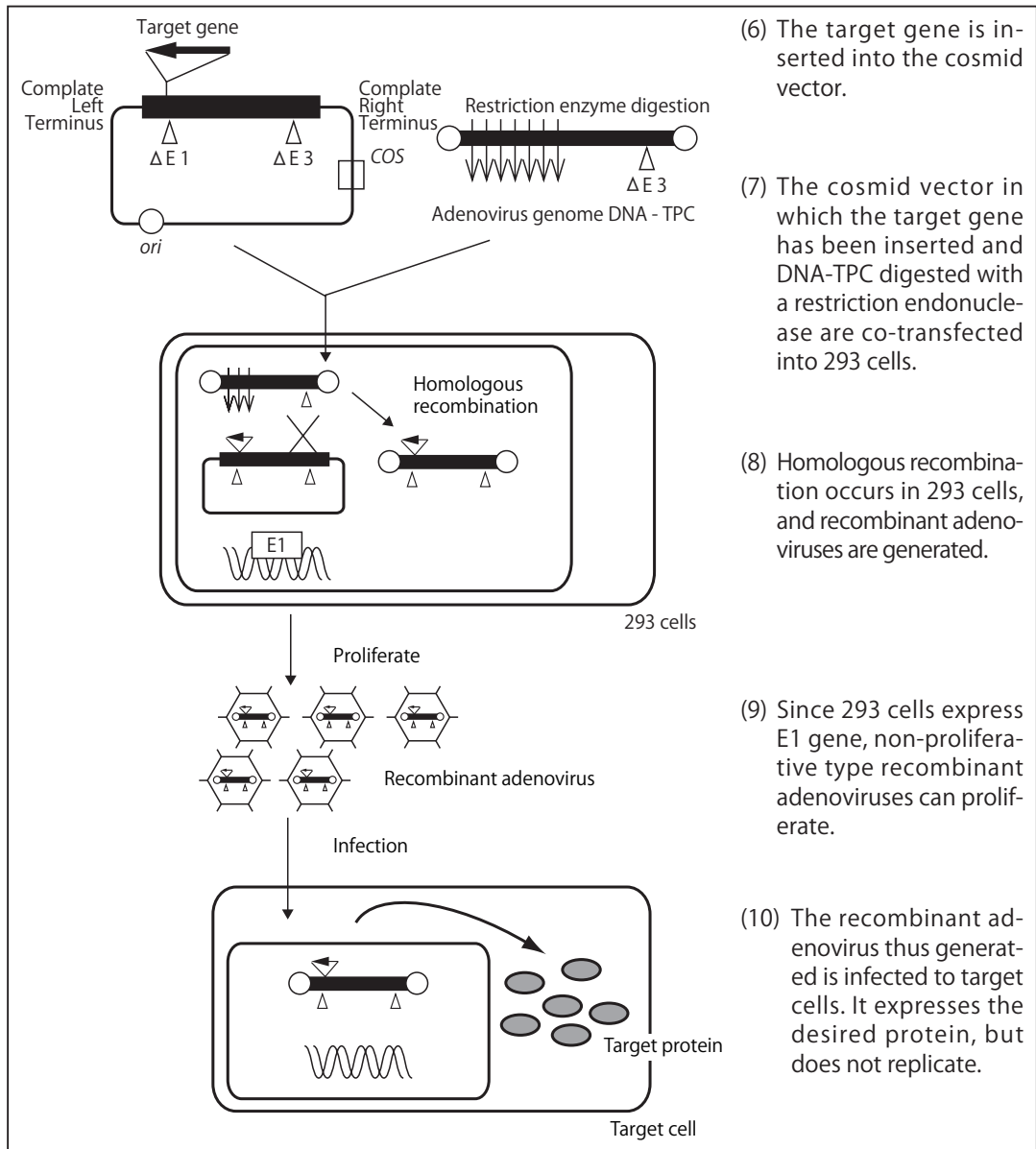


Fig. 1-1 Principles of generation of recombinant adenoviruses with "Full-length DNA transfer method".



(6) The target gene is inserted into the cosmid vector.

(7) The cosmid vector in which the target gene has been inserted and DNA-TPC digested with a restriction endonuclease are co-transfected into 293 cells.

(8) Homologous recombination occurs in 293 cells, and recombinant adenoviruses are generated.

(9) Since 293 cells express E1 gene, non-proliferative type recombinant adenoviruses can proliferate.

(10) The recombinant adenovirus thus generated is infected to target cells. It expresses the desired protein, but does not replicate.

Fig. 1-2 Principles of generation of recombinant adenoviruses with "COS-TPC method".



**IV. Content of the kit**

This kit contain cosmid vectors (Dual cosmid), and other necessary reagents to perform Full length transfer method. Adenovirus genome DNA-TPC is required further to perform COS-TPC method.

**IV-1. Kit Component**

Adenovirus Expression Vector Kit (Dual Version) Ver.2 (Cat.#6170)		
1. Cosmid Vector pAxcwit2 * 1	0.3 µg/µl	25 µl
2. Cosmid Vector pAxCAwtit2 * 2	0.3 µg/µl	25 µl
3. Restriction endonuclease <i>Smi</i> I	10 U/µl	20 µl
4. 10×H buffer		100 µl
5. Restriction endonuclease <i>Bsp</i> T104 I	10 U/µl	30 µl
6. 10×L buffer		100 µl
7. DNA Dissolution Buffer		50 µl
[ 100 mM Tris-HCl (pH7.5), 5 mM MgCl <sub>2</sub> , 300 mM NaCl ]		
8. Ligation Solution		50 µl
9. 10×TNE		2×1 ml
[ 500 mM Tris-HCl (pH7.5), 1 M NaCl, 100 mM EDTA ]		
10. Proteinase K	20 mg/ml	200 µl
11. 10% SDS		200 µl
12. Control Cosmid pAxCAiLacZit * 3	0.3 µg/µl	50 µl

\* 1 : Without promoter

\* 2 : Contains CAG promoter<sup>9)</sup>

\* 3 : pAxCAwtit with β-gal gene inserted

Storage :

10% SDS : Thawed at 37°C and stored at room temperature.

Others : - 20°C

The following reagents are available separately.

- *Smi* I and 10×H Buffer : *Smi* I (Cat.#1111A)

- *Bsp*T104 I and 10×L Buffer : *Bsp*T104 I (Cat.#1225A)

*Bst*T104 I has three isoschizomers; *Csp*45 I, *Nsp* V, and *Bst*B I. All of them have the same recognition sequence and cutting site.

- Ligation Solution : Enzyme Solution (B solution) of DNA Ligation Kit Ver.1 (Cat.#6021) can be used in replace it.

- Proteinase K : Proteinase K (Cat.#9033)

Separately available for COS-TPC method :

Adenovirus genome DNA-TPC (Cat.#6171)	
1. Adenovirus genome DNA-TPC	50 µl

Storage :

Store at -80°C.

**Note :** Since the efficiency of the Adenovirus genome DNA-TPC may be reduced by repeating freeze-thaw cycles, unnecessary freezing and thawing should be avoided.

#### IV-2. Cosmid vector structures

The Adenovirus Expression Vector Kit (Dual Version) Ver.2 (Cat.#6170) contains two types of dual cosmids, pAxcwit2 and pAxCAwtit2 (Figures 2-1 and 2-2), both of which are cosmid vectors containing the entire adenovirus genome, except for the E1 and E3 genes.

- pAxcwit2<sup>2)</sup>: a basic vector lacking a promoter and polyA sequence that allows for the insertion of up to about 7 kbp of an expression cassette (promoter + **target gene** + **poly (A) signal**).
- pAxCAwtit2<sup>2)</sup>: a vector containing CAG promoter (cytomegalovirus enhancer, chicken  $\beta$ -actin promoter, and rabbit  $\beta$ -globin poly (A) signal), which is a strong mammalian promoter<sup>9)</sup>, that allows for the insertion of up to about 5 kbp of a gene of interest.

Both vectors contain *Smi* I (*Swa* I) and *Cla* I cloning sites.

The constructed recombinant cosmids can be used with both the full-length DNA transfer and the COS-TPC recombinant adenovirus preparation methods. The structures of the pAxcwit2 and pAxCAwtit2 vectors are shown in Figures 2-1 and 2-2.

- ※ The sequence of cosmid vectors are available on the web :  
([http://www.takara-bio.com/products/download\\_e.htm](http://www.takara-bio.com/products/download_e.htm))

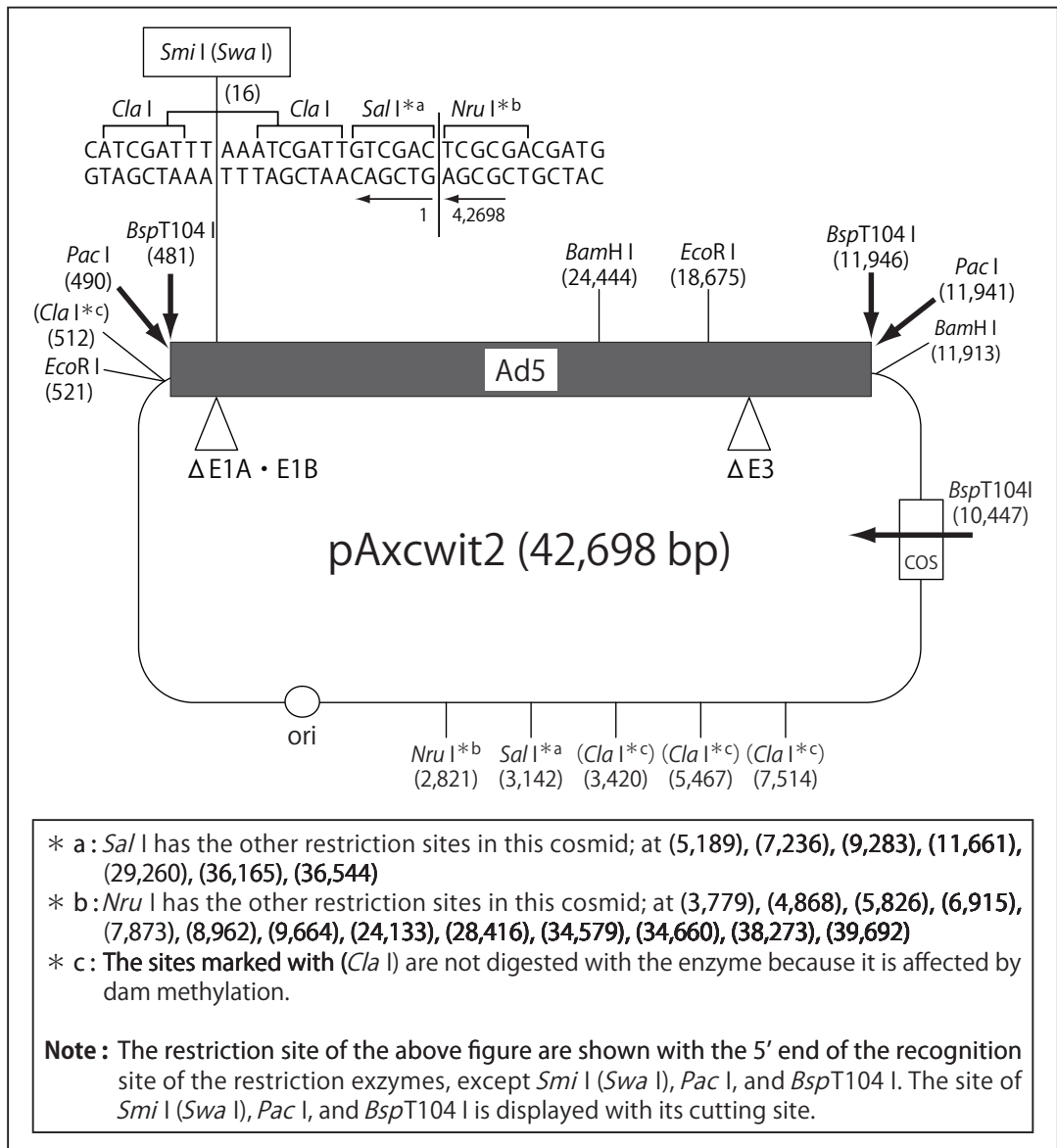


Fig. 2-1 Structure of cosmid vector pAxcwit2

pAxcwit2 is a basic vector and does not contain a promoter sequence. A foreign DNA of about 7 kbp can be inserted into *SmI I* or *Cla I* site.

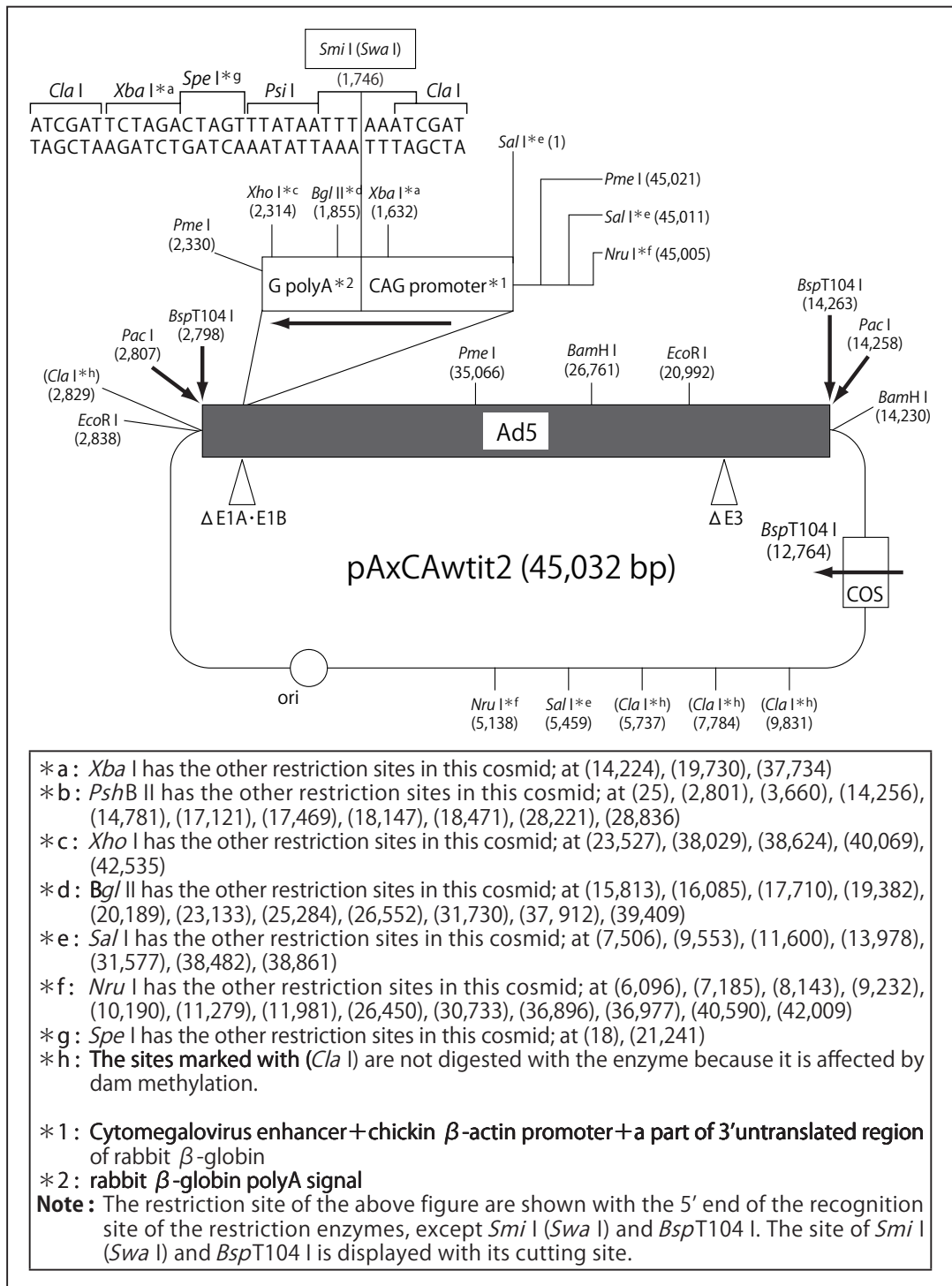


Fig. 2-2 Structure of cosmid vector pAxCAwtit2

pAxCAwtit2 contains highly efficient CAG promoter (cytomagalovirus enhancer, chicken  $\beta$ -actin promoter, rabbit  $\beta$ -globin poly A signal)<sup>2</sup> that act in mammals. A target DNA of about 5 kbp can be inserted into *Smi* I or *Cla* I site.

**V. Instruments and reagents required other than the kit****V-1. Instruments and equipments**

- Safety cabinet
- Humidified CO<sub>2</sub> incubator
- Microscope for cell observation
- High-speed micro-centrifuge
- Refrigerated centrifuge
- Sealed type sonicator (unnecessary for small-scale experiments)
- -80°C freezer
- Incubator (25~50°C)
- Electrophoretic apparatus
- Vortex mixer
- Autoclave sterilizer
- Autopipetter
- Sterilized pipettes
- Sterilized tips with filters
- Sterilized 50-ml centrifugation tubes
- Sterilized micro centrifugation tube
- Sterilized microtube with sealing cap (for stock of virus solution)
- Collagen-coated 96-well plates
- Collagen-coated 24-well plates
- Collagen-coated 25-cm<sup>2</sup> flasks
- Collagen-coated 75-cm<sup>2</sup> flasks
- Petri dishes for cell cultivation (ø 10 cm, ø 6 cm)
- 8-channel multi-pipettes
- Sterilized reserver for multi-pipettes

**V-2. Reagents**

- λ-packaging kit (eg. Gigapack® III XL packaging Extract (STRATAGENE))
- Transfection reagent (eg. *TransIT*® (Mirus Bio) )
- *recA*<sup>-</sup> transfection reagent *Escherichia coli* strain (eg. DH5 α)
- LB liquid medium + Ampicillin, LB plate + Ampicillin  
(Ampicillin concentration : 50~100 μg/ml)
- Phenol/chloroform
- Ethanol
- Sterilized distilled water
- TE buffer
- EDTA solution
- DNA Blunting Kit (Cat.#6025)
- Agarose for electrophoresis (eg. SeaKem® GTG Agarose (Lonza))
- Molecular weight Markers (eg. Wide-Range DNA Ladder (Cat.#3415A))
- 293 cells (eg. ATCC CRL-1573)
- HeLa cells (eg. ATCC CCL-2)
- Dulbecco's Modified Eagle's Medium (DMEM) (glucose 1 g/l)
- L-Glutamine
- Fetal Bovine Serum (FBS)
- Penicillin/Streptomycin
- 0.02% EDTA/PBS (-)
- RNase A
- Restriction endonuclease *Cla* I (Cat.#1034A)
- Restriction endonuclease *Xho* I (Cat.#1094A)
- Restriction endonuclease *Pac* I (NEB etc.) (if necessary)
- FastPure™ DNA Kit (Cat.#9191) (if necessary)
- Dry ice

## VI. Overview of Protocol

Operation	Protocol	Days required
Costruction and preparation of recombinant cosmid		4~5 days
↓ Insertion of an insert into cosmid vector.	A-1 (Page 16)	
↓ Confirmation of the structures of recombinant cosmid.	A-2 (Page 18)	
↓ Large-scale preparation of recombinant cosmid.	A-3 (Page 18)	
Preparation and Confirmation of recombinant adenovirus		About 3 weeks
↓ Generation of recombinant adenovirus.	B-1- (1) or B-1- (2) (Page 19 or 20)	
↓ Confirmation of recombinant adenovirus.	B-2 (Page 22)	
Preparation of high-titer recombinant adenovirus	C (page 24)	About 1 week
↓		
Quality check		
↓ [ viral titration 50% Tissue Culture Infectious Dose (TCID <sub>50</sub> ) ]	D (Page 25)	About 2 weeks
↓ [ RCA screening	Appendix 6 (Page 37)	About 1 week ]
Infection of adenovirus into target cells	E (Page 26)	

\* Each cosmid vector can be used to produce recombinant adenovirus through methods described in "VII. Protocol". The protocol to construct recombinant adenovirus should be referred to B-1- (1) in case with "Full length DNA Transfer method", and to B-1- (2) in case with "COS-TPC method".

## &lt; Important: Virus isolation &gt;

It is recommended that virus isolation is performed in the first step of recombinant adenovirus preparation. That protocol is below:

At next day after transfected with recombinant cosmid into 293 cells, the cells are harvested, diluted, and inoculated into 96 well plates.

The adenovirus from well in which cells are degenerated completely after 8 days incubation in CO<sub>2</sub> incubator is used as a primary virus stock, after cultured for 18 days. Single clone of recombinant adenovirus will be obtained at high probability by performing these steps, though it is required approximately 2.5 weeks (3 weeks in included confirmation of its structure).

The virus isolation should be always performed as described in this protocol. In the COS-TPC method, the virus isolation is important to avoid contamination of parent or unwanted viruses resulting from unexpected homologous recombination. In the full-length DNA transfer method, there is not a risk of parent virus contamination theoretically in a virus stock. However, the adenovirus having deletion in a part of the gene of interest will appear at very low frequency. Even though the virus with deletion is present at a trace amount, there is probability that the deleted virus become majority in virus stock during large scale preparation of the stock. Therefore, it is not recommended to use the virus stock directly amplified after transfection. It is also important to verify that there are no problem in each step of the protocol. The protocol shows to confirm a structure of recombinant virus at the secondary virus preparation (VII. B-2 Confirmation of the recombinant adeno-

virus) and quaternary virus preparation (VII. C Preparation of high-titer recombinant adenovirus) steps. Especially, in case to apply to animal experiment etc. which required a significant investment of time and effort, it is recommended to prepare and purify high-quality recombinant adenovirus preparation according to this protocol for obtaining reliable and secure results.

#### < Control experiment >

When preparing recombinant adenoviruses, it is strongly recommended that control experiments is simultaneously performed (See section VIII. Q&A. Q4). The Adenovirus Expression Vector Kit (Dual Version) Ver.2 contains the control cosmid pAxCaILacZit, which was produced by inserting the *Escherichia coli*  $\beta$ -galactosidase gene (*lacZ*) into the *Smi* I (*Swa* I) site of the cosmid vector pAxCaWtit2 and allows for the production of the recombinant adenovirus AxCAiLacZ by either the full-length DNA transfer or COS-TPC methods. AxCAiLacZ expresses the  $\beta$ -galactosidase gene (*lacZ*), allowing its use in determination of conditions for infection into target cells as well (VII. E Infection of adenovirus into target cells).

## VII. Procedure

### A. Construction and Preparation of the recombinant cosmid.

#### A-1. Insertion of a target DNA into the cosmid vector

To obtain recombinant adenoviruses, first, an insert DNA is inserted into cosmid vectors. Both of cosmid vectors contain *Smi* I (*Swa* I) and *Cla* I cloning site, target DNA is usually inserted into *Smi* I site after the terminal of the target DNA is converted to blunt end.

(It is possible to use *Cla* I cloning site after cutting both the vector and a target DNA with *Cla* I.)

1. Prepare about 0.5  $\mu$ g of insert DNA fragment. If the terminals are not blunt, convert them to blunt-end, ex. by using DNA Blunting Kit (Cat.#6025).
2. Purify the insert DNA fragment through phenol/chloroform extraction and ethanol precipitation.
3. Cut cosmid vector completely with the restriction endonuclease *Smi* I (*Swa* I). Prepare the following reaction mixture and incubate at 30°C for 2 hours.

Cosmid vectors	5 $\mu$ l
10×H Buffer	5 $\mu$ l
<i>Smi</i> I	2 $\mu$ l
Sterilized distilled water	up to 50 $\mu$ l

4. After adding EDTA to a final concentration of 10 mM, purify the digested cosmid vectors by phenol/chloroform extraction.
5. Add 0.1~0.2  $\mu$ g of the insert DNA prepared in A-1-2 to the cosmid vector above in A-4.
6. Perform ethanol precipitation. Since the cosmid DNA becomes insoluble once it is dried, immediately perform the next process simply by removing residual ethanol, ex. by wiping up residual ethanol with the tip of a non-fibrous paper.
7. Ligate the insert DNA fragments and digested cosmid vectors. Dissolve them by adding 5  $\mu$ l of DNA Dissolution Buffer and add 5  $\mu$ l of the Ligation Solution. Incubate the mixture at 25°C for 10 minutes.
8. Perform ethanol precipitation. Do not allow DNA to dry.

9. Cut DNA with the restriction endonuclease *Smi* I (*Swa* I). Add the following reagents to the ligated DNA and incubate it at 30°C for 2 hours.

10×H Buffer	5 μl
<i>Smi</i> I	2 μl
Sterilized distilled water	up to 50 μl

- \* This reaction is important to prevent the appearance of cosmids without inserts. However, this process cannot be performed if the *Smi* I site is present in the insert.

10. Package an appropriate amount of DNA using a λ-packaging kit. It will be easy to select the cosmid by the use of a kit that selectively packages relatively large DNA (eg. Gigapack® III XL packaging Extract (STRATAGENE)).

- \* Follow the manufacturer's instructions for the use of packaging kits. An example of usage is shown below as a reference.

- 1) Preparation of 2×bacterial cultures. At first, Plate *recA*<sup>-</sup> *E. coli* (e.g., DH5 α). At next day, pick single colony and culture in LB broth overnight at 37°C with agitation. Inoculate 5 ml of LB broth supplemented with 0.2% maltose with 50 to 100 μl of the overnight culture and incubate at 37°C with agitation until the OD<sub>650</sub> reaches approximately 1.0 (4~6 hours).

Take 1 ml of the culture in a sterile tube and remove the supernatant by centrifugation.

Suspend the pellet (bacteria) in 500 μl of 10 mM MgSO<sub>4</sub> to prepare 2×bacterial cultures.

- 2) Packaging : Mix 1 μl of the DNA digest obtained in A-1-9 and packaging extract and incubate at room temperature (22°C) for 1.5 hours.

- 3) Add 100 μl of SM buffer \* to the mixture prepared in 2).

- \* SM buffer : Mix 5.8 g of NaCl, 2.0 g of MgSO<sub>4</sub> · 7H<sub>2</sub>O, 50 ml of 1 M Tris-HCl (pH7.5), and 5.0 ml of 2% (w/v) gelatin in distilled water, add distilled water to a final volume of 1 L, and autoclave.

- 4) Infection : Mix 100 μl of the solution prepared in 3) and 100 μl of the 2×bacterial cultures prepared in 1) and incubate at room temperature for 10 minutes.

- 5) Add 1 ml of LB broth and let stand at 37°C for 20 minutes.

11. Seed 1/100, 1/10, and the rest of the infected *Escherichia coli* on agar plates containing ampicillin, and incubate them overnight at 37°C.

12. Pick up the whole colony, incubate it in 1.5 ml LB + amp., and prepare cosmid DNA.

- \* Do not prepare stock in the *Escherichia coli*, since the cosmid is lost when it is subcloned over more generations in *Escherichia coli*.

- \* If the cosmid vectors are digested with *Smi* I after ligation, testing of around 10 colonies will be sufficient to obtain target clones since most clones are likely to contain the insert DNA. If *Smi* I digestion is not performed, testing of 24 to 36 colonies is typically sufficient to obtain target clones.

13. Confirm the direction and structure of the insert DNA by treatments with restriction endonucleases or other methods.

(Refer to the A-2. Confirmation of the structure of the recombinant cosmid.)



**A-2. Confirmation of the structures of the recombinant cosmid.**

Since recombinant virus preparation is a time-consuming process, it is important to make sure that the true recombinant cosmids have been constructed.

1. Select cosmid clones that contain the insert. The insert can be cut out by *Cla*I digestion.
2. Confirm the orientation of the insert by digestion with appropriate restriction enzymes (IV-2. Cosmid vector structures).
  - \* Plasmids lacking most of the adenovirus genome can be obtained by restriction digestion with *Nru*I or *Sal*I and then ligation ("deadenization", Figure 3). The plasmids obtained in this manner ("deadenized plasmids") can also be used to confirm structures in cases in which no appropriate restriction sites are available.
  - \* The complete sequences of the cosmid vectors are available at the TaKaRa website.
3. At this point, expression of the target gene should be verified by transfecting the obtained recombinant cosmids or deadenized plasmids into cells using an appropriate transfection method.
  - \* Deadenized plasmids are useful as expression plasmids as well.

**A-3. Large-scale preparation of the recombinant cosmid.**

Recombinant cosmids will be amplified to prepare the amount required for recombinant adenovirus preparation.

Since cosmids will be deleted in *E. coli* during extended passaging, they should be stored as DNA after amplification using the following method.

1. Mix 0.3 to 0.5  $\mu$ g of cosmid (circular) DNA and  $\lambda$  packaging extract, and perform packaging as described in A-1-10.
2. Plate a 1/100 volume of the mixture onto an LB-ampicillin agar plate and incubate overnight at 37°C. Add the remaining mixture to 50 ml of LB broth containing ampicillin and incubate overnight at 37°C with agitation.
3. On the following day, if 10 or more colonies per plate are detected, prepare cosmid DNA from 50 ml of the liquid culture. If less than 10 colonies per plate are detected, discard the liquid culture and repeat the procedure, since the number of passages would be too high even if the culture contained *E. coli*.
  - Typically, 50 ml of culture yields 50 to 100  $\mu$ g of cosmid DNA.
  - If using the COS-TPC method for virus preparation, purify a sufficient purity of cosmid DNA for transfection using CsCl density-gradient centrifugation method or an appropriate DNA purification kit and dissolve in sterile distilled water.
4. If using the full-length DNA transfer method for virus preparation, follow the instructions at <1> Full-length DNA transfer method in section B-1 Generation of the Recombinant adenovirus after cosmid DNA preparation. If using the COS-TPC method for virus preparation, follow the instructions in section B-1-<2> COS-TPC method after cosmid DNA preparation.
  - Use of the full-length DNA transfer method requires 15  $\mu$ g of recombinant cosmid for each experiment.
  - Use of the COS-TPC method requires 8  $\mu$ g of recombinant cosmid for each experiment.

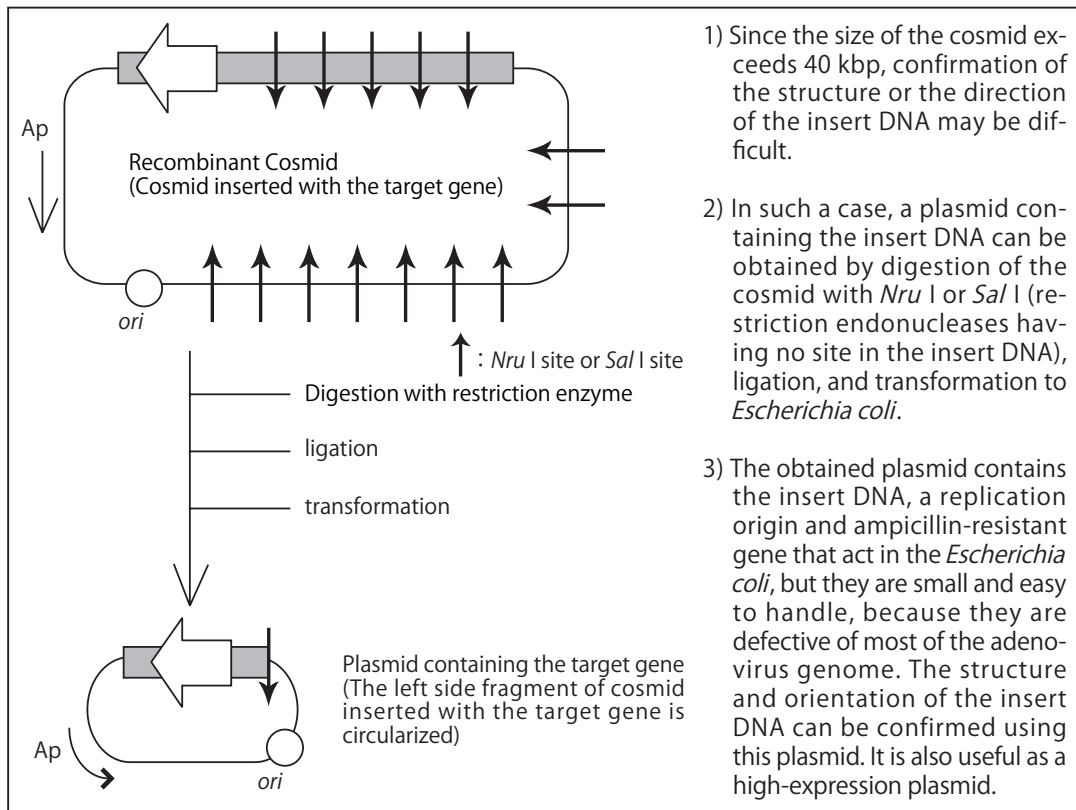


Fig. 3 Deadenization

**B. Generation and Confirmation of the recombinant adenovirus**

The 293 cells are an essential component in recombinant adenovirus preparation. Refer to Appendix 1 for maintenance and precautions for 293 cells and to Appendix 2 for handling and precautions for recombinant adenoviruses.

**B-1. Generation of recombinant adenovirus**

**B-1-<1> Full-length DNA transfer method**

**Note :** In case that a gene of interest contains neither *Bsp*T104 I (TTCGAA) or *Pac* I (TTAATTAA) recognition site, the recombinant cosmid DNA should be cut with *Bsp*T104 I, of which sites are located nearer terminal ends of adenovirus DNA. In only case that a gene of interest contains *Bsp*T104 I site but not *Pac* I, the *Pac* I digestion is useful. The full-length DNA transfer method cannot be used if a gene of interest contains both *Bsp*T104 I and *Pac* I sites. In that case, the COS-TPC method (B-1-<2>) must be used.

1. Digest the recombinant cosmid DNA prepared in A-3 with the restriction enzyme *Bsp*T104 I. Prepare the following reaction solution and incubate at 37°C for 2 hours.

Recombinant cosmid DNA	15 μg
<i>Bsp</i> T104 I (10 U/μl)	5 μl
10×L Buffer	10 μl
Sterilized distilled water	up to 100 μl

2. Extract once with phenol/chloroform and twice with chloroform, precipitate with ethanol, and dissolve the DNA pellet in 30  $\mu$ l of sterile distilled water.
3. Run 1  $\mu$ l of the DNA on an agarose gel to confirm complete digestion with *Bsp*T104 I.  
Three bands with sizes of 1.49, 9.96, and  $\geq$  30 kbp \* will be visible if digestion is complete. Determine DNA yield by comparing the intensity of the 1.49 kbp band with the intensity of DNA of known concentration.  
\* : For example, insertion of a 1 kbp fragment into pAxCAwtit2 will produce a band of 34.6 kbp (See Figures 2-1 and 2-2).
4. Prepare two 60-mm cell culture plates of 293 cells.
  - One plate will be used in B-1-<1>-5 and the other in B-1-<1>-7. Prepare the cells so that they will be 100% confluent at the time of each use.
5. Transfect 10  $\mu$ g of the *Bsp*T104 I digested cosmid into 293 cells using either lipofection or calcium phosphate precipitation methods.
  - An example of transfection using *TransIT*<sup>®</sup>-293 (Mirus Bio) is shown below as a reference.
    - 1) Remove the medium from 60-mm plates and add 2.5 ml of serum-free medium (e.g., Opti-MEM, Invitrogen).
    - 2) Mix 15  $\mu$ l of *TransIT*<sup>®</sup>-293 Reagent with 250  $\mu$ l of the serum-free medium by vortexing and incubate at room temperature for 5 minutes.
    - 3) Add sterile distilled water to 10  $\mu$ g of the digested recombinant cosmid DNA prepared in B-1-<1>-2 to a final volume of 30  $\mu$ l.
    - 4) Add the DNA solution prepared in 3) to the mixture prepared in 2), mix gently, and incubate at room temperature for 5 minutes.
    - 5) Add the DNA suspension prepared in 4) to the plates prepared in 1) in a dropwise fashion and swirl the plates gently to disperse the DNA suspension evenly. Incubate the plates at 37°C in a CO<sub>2</sub> incubator.
6. In the next morning, harvest the cells using EDTA-PBS (–) in the same manner as for passaging.
  - If the cells do not detach readily, use trypsin at a lower than usual concentration (approximately 0.025% ).
7. Plate the cell suspension and 10-fold diluted cell suspension onto collagen-coated 96-well plate each. To achieve a consistent number of cells in each plate, mix nontransfected 293 cells grown on a 60-mm plate and the 10-fold diluted cell suspension at the following ratio and add 100  $\mu$ l of the cell mixture per well.

Transfected 293 cells in the 60-mm plate → suspend in 11 ml of medium : (A)  
Nontransfected 293 cells in the 60-mm plate → suspend in 11 ml of medium : (B)  
10-fold dilution plate = 1 ml (A) + 10 ml (B)  
Undiluted plate = 10 ml (A)

8. Add 50  $\mu$ l of 10% FCS-DMEM to each well after 5 to 6 days and after 10 to 11 days, changing tips for each well.
  - Use sterile filter tips to avoid contamination and cross-contamination of virus samples.
9. Evidence of cytopathic effects resulting from virus propagation are visible in some wells in 7 to 15 days. Transfer cells with medium from each well in which all cells have degenerated into 1.5-ml sterile tubes under sterile conditions, freeze quickly in dry ice, and store at  $-80^{\circ}\text{C}$ .
  - As viruses propagate, cells lose their ability to adhere and appear round and floating (Appendix 1-6). Transfer cells when all cells in a well reach this state.
10. The assay will be completed in 15 to 18 days. Select preferentially about 4 tubes containing cultures collected from wells that showed completely degeneration of cells in a relatively late period (at 8 days or later) (B-1-<1>-9).
  - Do not select wells in which virus propagation occurred in a relatively early period, as they may contain multiple virus clones.
11. Lyse cells by 6 rounds of freeze-thaw; i.e., freeze quickly in dry ice and thaw in a  $37^{\circ}\text{C}$  water bath.
12. Collect the supernatant by centrifugation at 5,000 rpm for 5 minutes at  $4^{\circ}\text{C}$  and store at  $-80^{\circ}\text{C}$  as the first virus stock.

#### B-1-<2> COS-TPC method

1. Prepare 293 cells cultured on each one 60-mm and one 100-mm plate.
  - The 60-mm plate will be used in B-1-<2>-2 and the 100-mm plate in B-1-<2>-4. Prepare the cells so that they will be 100% confluent at the time of each use.
2. Co-transfect 8  $\mu$ g of the recombinant cosmid DNA prepared in A-3 and 10  $\mu$ g of Adenovirus genome DNA-TPC (Cat.#6171) into 293 cells cultured on a 60-mm plate by either lipofection or calcium phosphate precipitation methods.
  - Restriction digestion of recombinant cosmids is not required.
  - An example of transfection using *TransIT*<sup>®</sup>-293 (Mirus Bio) is shown below as a reference.
  - 1) Remove the medium from 60-mm plates and add 2.5 ml of serum-free medium (e.g., Opti-MEM, Invitrogen).
  - 2) Mix 15  $\mu$ l of *TransIT*<sup>®</sup>-293 Reagent with 250  $\mu$ l of the serum-free medium by vortexing and incubate at room temperature for 5 minutes.
  - 3) Add sterile distilled water to a mixture of 8  $\mu$ g of the recombinant cosmid DNA and 10  $\mu$ l of Adenovirus genome DNA-TPC to a final volume of 30  $\mu$ l.
  - 4) Add the DNA solution prepared in 3) to the mixture prepared in 2), mix gently, and incubate at room temperature for 5 minutes.
  - 5) Add the DNA suspension prepared in 4) to the plates prepared in 1) in a dropwise fashion and swirl the plates gently to disperse the DNA suspension evenly. Incubate the plates at  $37^{\circ}\text{C}$  in a  $\text{CO}_2$  incubator.

3. In the next morning, harvest the cells using EDTA-PBS (–) in the same manner as for passaging.
  - If the cells do not detach readily, use trypsin at a lower than usual concentration (approximately 0.025% ).
4. Plate the undiluted, 10-fold diluted, and 100-fold diluted cell suspensions onto collagen-coated 96-well plate each. To achieve a consistent number of cells in each plate, mix nontransfected 293 cells grown on a 100-mm plate to the 10-fold and 100-fold diluted cell suspensions at the following ratio and add 100  $\mu$ l of the cell mixture per well.

Transfected 293 cells in 60-mm plate → suspend in 11 ml of medium : (A)  
Nontransfected 293 cells in 100-mm plate → suspend in 30 ml of medium : (B)  
100-fold diluted plate = 0.1 ml (A) + 11 ml (B)  
10-fold diluted plate = 1 ml (A) + 10 ml (B)  
Undiluted plate = 10 ml (A)

5. Add 50  $\mu$ l of 10% FCS-DMEM to each well after 5 to 6 days and after 10 to 11 days, changing tips for each well.
  - Use sterile filter tips to avoid contamination and cross-contamination of virus samples.
6. Evidence of cytopathic effects resulting from virus propagation are visible in some wells in 7 to 15 days. Transfer cells with medium from each well in which all cells have degenerated into 1.5-ml sterile tubes under sterile conditions, freeze quickly in dry ice, and store at –80°C.
  - As viruses propagate, cells lose their ability to adhere and appear round and floating (Appendix 1-6). Transfer cells when all cells in a well reach this state.
7. The assay will be completed in 15 to 18 days. Select about 10 tubes containing cultures collected from wells that showed cell degeneration in a relatively late period (at 8 days or later) (B-1-<2>-6.) from plates containing approximately 10 wells in which virus propagation (cell degeneration) is visible.
  - Do not select wells in which virus propagation occurred in a relatively early period, as they may contain multiple virus clones.
8. Lyse cells by 6 rounds of freeze-thaw; i.e., freeze quickly in dry ice and thaw in a 37°C water bath.
9. Collect the supernatant by centrifugation at 5,000 rpm for 5 minutes at 4°C and store at –80°C as the primary virus stock.

### **B-2. Confirmation of the recombinant adenovirus**

1. Prepare cultures of 293 cells and HeLa cells that are 70% to 100% confluent on collagen-coated 24-well plates.
2. Infect the cells with each sample of primary virus stock using 2 wells for the 293 cells and 1 well for the HeLa cells. Remove the medium and add 10  $\mu$ l of primary virus stock and 0.1 ml of 5% FCS-DMEM to each well.
  - Care should be taken not to let allow the cells to dry out during the procedures.
3. Infect the cells by swirling the plates gently to disperse the virus stock solution evenly. Carry out the swirling 3 to 4 times every 15 to 20 minutes. The cells should be kept in a CO<sub>2</sub> incubator (37°C, 5% CO<sub>2</sub>) during this time.

4. After 1 hour of infection, add 0.4 ml of 5% FCS-DMEM.
5. After 3 days, select virus clones that caused no degeneration in HeLa cells and complete degeneration in 293 cells.
  - The recombinant adenovirus should not replicate in cells other than 293 cells. Virus clones that show cytopathic effects in HeLa cells should be excluded at this point, because they may be contaminated with viruses that carry the E1 gene.
6. Collect cells and medium from a well containing 293 cells infected with each virus sample and subject to 6 rounds of freeze-thaw.
7. Collect the supernatant by centrifugation at 5000 rpm for 5 minutes at 4°C, transfer to screw-cap microcentrifuge tubes, freeze quickly in dry ice, and store at -80°C (secondary virus stock).
  - Screw-cap microcentrifuge tubes (e.g., tubes with O-rings) are recommended for storage of virus stocks to prevent contamination and for safer handling.
8. Collect the cells and medium from another well containing 293 cells, remove the supernatant by centrifugation at 5000 rpm for 5 minutes at 4°C, and store the cell pellet at -80°C (cell pack).
9. Extract all DNA from the cell pack using the following method and confirm the structure of the recombinant adenovirus DNA.
 

It is possible to easily, rapidly extract DNA by using FastPure™ DNA Kit (Cat.#9191) in place of Step 10~15 below.

  - Recombinant viruses produce 10,000 replicates per cell in 293 cells, allowing for easy verification of recombinant virus structures by restriction digestion of DNA extracted from infected cells.
10. Add the following reagents to the cell pack to a total volume of 400 μl.

10×TNE	40 μl
Proteinase K (20 mg/ml)	4 μl
Sterile distilled water	up to 400 μl

11. Suspend the cell pack by vortexing.
12. Add 4 μl of 10% SDS and mix well by vortexing.
13. Incubate at 50°C for 1 hour.
14. Extract twice each with phenol/chloroform and chloroform.
  - Mix well by vortexing.
15. Precipitate with ethanol and dissolve the DNA pellet in 50 μl of TE Buffer containing 20 μg/ml RNase A.
  - It may be difficult to dissolve the DNA pellet if allowed to dry completely following ethanol precipitation.
16. Cut the insert DNA by digesting 15 μl DNA suspension with the appropriate restriction enzymes and confirm the desired restriction pattern by agarose gel electrophoresis. At the same time, confirm the left-side fragment by *Cla*I or *Xho*I digestion.
  - Confirmation of electrophoresis pattern.

	<i>Cla</i> I reaction	<i>Xho</i> I reaction
Extracted DNA	15 μl	15 μl
Restriction enzyme	2 μl (about 2 U)	2 μl (about 2 U)
10 × buffer	2 μl	2 μl
Sterile dH <sub>2</sub> O	up to 20 μl	up to 20 μl
Incubation	30°C, 2 hours	37°C, 2 hours

- Confirmation of the left-side fragment (see also Figure 2 and Appendix 7) :

Cosmid vector	<i>Cla</i> I digestion	<i>Xho</i> I digestion
pAxcwit2	0.46 kbp	
pAxCawtit2	1.0 kbp	0.48 kbp
Wild type	0.92 kbp	5.8 kbp

- With pAxCawtit2, the size of the left-side fragment produced by *Cla* I digestion is 1.0 kbp, which could be misidentified as the 0.92 kbp wild-type fragment; therefore, it is recommended that *Xho* I digestion is performed as well.
- Insert DNA can be verified by *Cla* I digestion in the same manner as described in section A-2. Confirmation of the structure of the recombinant cosmid.
- A cosmid DNA containing the insert digested with *Cla* I as a control should be run in the electrophoresis for the confirmation. Select clones producing the bands correct restriction pattern for the cosmid vector and the left-side fragment of the adenovirus genome.
- Do not select clones producing unexpected faint bands since they may be contaminated by viruses with deletions.
- If the determination is difficult due to the presence of cellular genomic DNA, use genomic DNA extracted from uninfected 293 cells as a negative control.

### C. Preparation of high-titer recombinant adenovirus

1. Prepare cultures of 293 cells that are 70% to 100% confluent in 2 collagen-coated 25 cm<sup>2</sup> flasks.
2. Infect the cells with the secondary virus stock for the target viruses selected in the analysis performed in B-2. Gently add 15  $\mu$ l of virus stock and 0.5 ml of 5% FCS-DMEM into the flasks from which medium has been removed beforehand.
3. Slowly swirl the flasks to disperse the virus evenly to all cells. Perform this step 3 to 4 times every 15 to 20 minutes. The cells should be kept in a CO<sub>2</sub> incubator (37°C, 5% CO<sub>2</sub>) during this time.
4. Infect for 1 hour and add 4.5 ml of 5% FCS-DMEM.
5. After all the cells show cytopathic effects (3-4 days), collect the cells with medium in sterile tubes under sterile conditions and disrupt the cells by freeze-thaw cycling or using a sealed type sonicator to release the viruses.
  - The use of a sealed type sonicator is recommended for handling large volumes. Do not use open sonicators as they generate aerosols.
6. Collect the supernatant by centrifugation at 3000 rpm for 10 minutes at 4°C. Dispense 1 ml each into 5 screw-cap microcentrifuge tubes, freeze quickly in dry ice, and store at -80°C (tertiary virus stock).
7. Infect 293 cells that are 70% to 100% confluent in 2 collagen-coated 75-cm flasks using 50  $\mu$ l of tertiary virus stock and 2 ml of 5 % FCS-DMEM in the same manner as described in C-3.
8. Infect for 1 hour and add 13 ml of 5% FCS-DMEM.
9. After all the cells show cytopathic effects (3-4 days), prepare the virus stock in the same manner as for the tertiary virus stock.
10. Dispense 1 ml each into 15 screw-cap microcentrifuge tubes, freeze quickly in dry ice, and store at -80°C (quaternary virus stock). Quaternary virus stocks are the virus stocks actually used in experiments and should

- produce high titers (approximately  $10^9$  pfu/ml).
11. Divide 0.1-ml aliquots from 1 tube of the quaternary virus stock at the first use, freeze quickly in dry ice, and store at  $-80^{\circ}\text{C}$  (working stock). Avoid repeated freezing and thawing.
  12. Infect 293 cells in 1 well of the 24-well plate using  $5\ \mu\text{l}$  of quaternary virus stock and verify the restriction patterns of the DNA of the propagated viruses using the methods described in B-2.
    - The presence of viruses with deletions or parent viruses at this point is likely attributable to a minute amount of viruses present in the secondary virus stock that have become visible as a result of rapid replication. Discard all the pertinent tertiary and quaternary virus stocks and either repeat virus stock preparation using a different secondary virus stock or clone target viruses from the primary virus stock by limiting dilution.
    - Virus stocks that will be used for infection should be tested at each passage to verify that no viruses with deletions or parent viruses have been produced (Appendix 6).

#### D. Viral titration [ 50% Tissue culture infectious dose (TCID<sub>50</sub>) method ]

The titer is assayed usually by observation of plaque formation on an agar medium (plaque forming method), but the results of this method are close consistent with those of the TCID<sub>50</sub> method described below.

1. Culture 293 cells in a 10-cm Petri dish.
2. Dilute the viral solution 10-fold serially using 5 % FCS-DMEM and prepare a  $10^4$  dilution of the viral solution. For example, dilute 0.1 ml of a viral solution with 0.9 ml of 5% FCS-DMEM.
3. Add  $50\ \mu\text{l}$  each of 5% FCS-DMEM in all wells of a collagen-coated 96-well plate.
4. Add  $25\ \mu\text{l}$  of a  $10^4$ -fold dilution of recombinant virus to each well of the first left column.
5. Transfer  $25\ \mu\text{l}$  to the wells of the second column using an 8-channel multi-pipette. Repeat this process to the 11th column and discard the last  $25\ \mu\text{l}$ . This results in serial dilutions of  $3^n$  up to  $3^{11} \times 10^4$ . Place only uninfected cells in the 12th column as a control. Use a new tip for each dilution.
6. Suspend the 293 cells (D-1) in 6 ml of 5% FCS-DMEM.
7. Add  $50\ \mu\text{l}$  of the cell suspension prepared in D-6 to each well.
8. Add  $50\ \mu\text{l}$  of 10% FCS-DMEM gently to each well after 4~5 days and 7~8 days.
9. Judge the end point of complete cytopathic effect by microscopy after 11~13 days. The cells of the wells in which the virus is present show cytopathic effect. The judgment is easy if the cells can be maintained until day 14, but becomes difficult if the cells are damaged.
10. Calculate the 50% tissue culture infectious dose (TCID<sub>50</sub>) statistically using Käber's formula. The values of TCID<sub>50</sub> calculated by this method using 293 cells are close consistent with the values of PFU determined by the plaque forming method. An example is shown in Figure 4.

$$\text{TCID}_{50} = (\text{dilution rate in the first column}) \times (\text{dilution rate})^{\Sigma - 0.5}$$

in which

$$\Sigma = \frac{\text{total sum of (number of wells showing cytopathic effect)}}{\text{(number of wells) at each step of dilution.}}$$



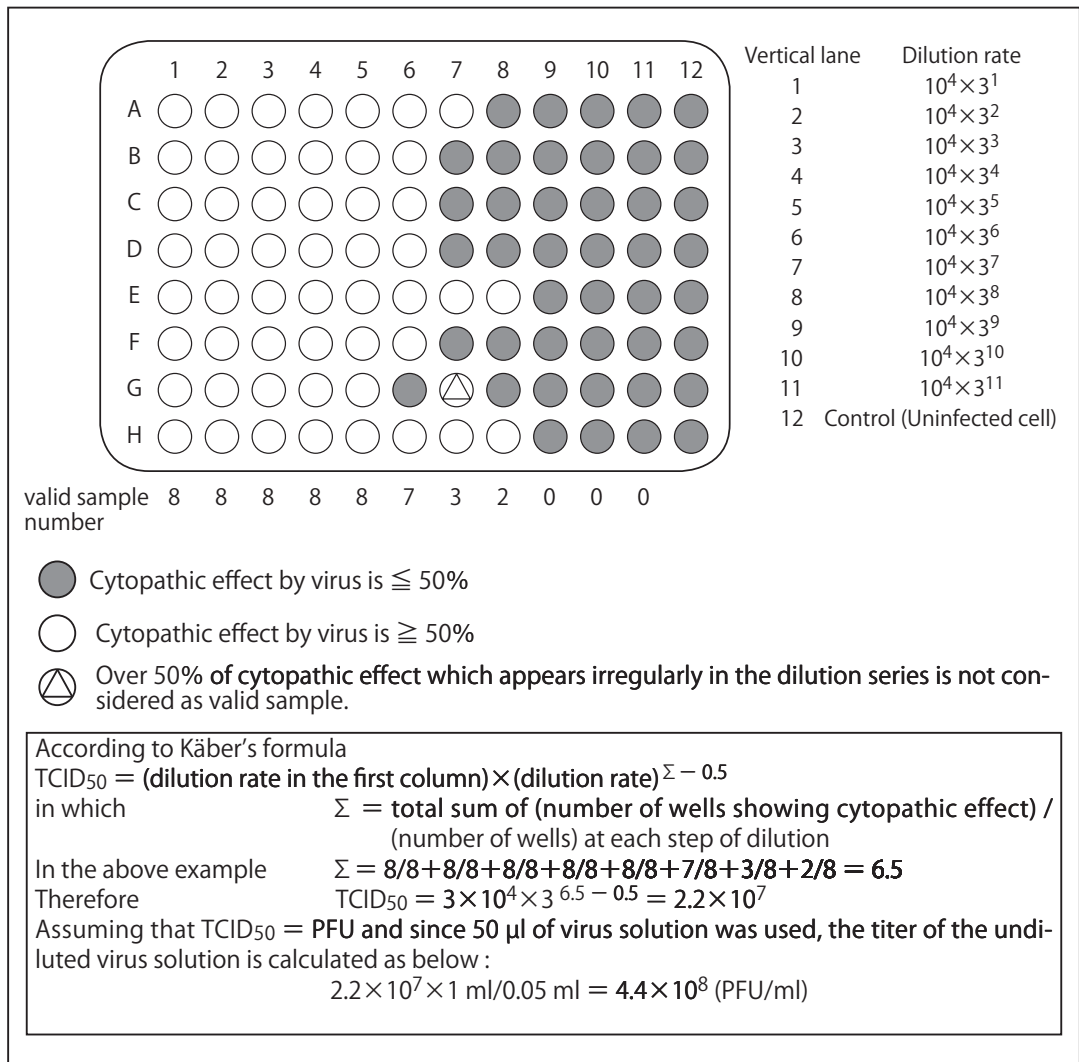


Fig. 4 Example of TCID<sub>50</sub> (Tissue Culture Infectious Does method.)

### E. Infection of target cells with the virus

A popular method to infect adherent cells is described. In the case of non-adherent cells, the cells are collected by centrifugation, suspended with a virus solution, and treated with the same procedure.

1. Prepare 5 or more wells, in the numbers of dilutions shown below, of cell cultures (70~100% confluent) for each virus.
2. Remove the medium. If the serum contained in the medium is not FCS (e.g. CS), wash the cells twice with a serum-free medium and remove the medium.
3. Add the undiluted quaternary virus stock and its 3-fold, 10-fold, 20-fold, and 40-fold diluents with a serum-free medium or a medium containing FCS in a plate. The recommended volume is 30~40  $\mu\text{l}$  in a 96-well plate, 50~70  $\mu\text{l}$  in a 24-well plate, and 100~200  $\mu\text{l}$  in a 10-cm culture dish.

4. Slowly swirl the plate several times to disperse the virus suspension to all cells to ensure infection, and then place the plate in a CO<sub>2</sub> incubator (37°C, 5% CO<sub>2</sub>). Repeat the process 3 ~ 4 times at 15 ~ 20 minute intervals.
5. At 1 hour after infecting with the quaternary virus stock, add an appropriate volume of the culture medium, and culture the cells at 37°C in a CO<sub>2</sub> incubator.
  - Infection time is usually sufficient for 1 (~2) hour.
6. Examine the expression of the target protein by an appropriate method such as immunofluorescent staining or Western blotting on the day of infection, and at 1, 2 and 3 days.

## VIII. Q & A

Q1. How should I choose a recombinant virus preparation method ?

A1. The choice of method depends on the characteristics of the gene of interest.

The first step is to construct recombinant cosmids by cloning the target gene into one of the cosmid vectors (pAxcwit2 or pAxCawtit2) and produce recombinant viruses with the full-length DNA transfer method. This method is sufficient for recombinant adenovirus preparation in most cases. Target viruses may be difficult to obtain in some cases, however, likely due to low efficiency of virus preparation attributable to factors associated with the products of the target gene, transfection efficiency, and the condition of 293 cells. In these cases, try the COS-TPC method using the same recombinant cosmid and Adenovirus genome DNA-TPC (Cat.#6171; Optional).

**Note :** If the insert DNA contains the *Bsp*T104 I or *Pac* I recognition site, the full-length DNA transfer method cannot be used and the COS-TPC method must be used instead.

**Note :** Perform a control experiment using the control cosmid pAxCaILacZit, since it aids in determining whether the causes for low efficiency of or no recombinant virus production lie in the target gene or the experimental conditions (e.g., transfection efficiency and conditions of 293 cells) (see section VI. < Control experiment > and VIII. Q&A. Q4).

Q2. Is it necessary to confirm expression of the recombinant cosmid ?

A2. A confirm experiment is recommended.

Recombinant adenovirus preparation is a time-consuming process. Thus, performing a confirmation experiment at each step, leads to more reliable results in the end.

Transfect target cells with a recombinant cosmid or a deadenized plasmid created from the recombinant cosmid (see Figure 3) and verify gene expression using an appropriate method. Other cells that allow for high transfection efficiency (e.g., 293 cells) can be used in place of the target cells in cases where transfection into the target cells is difficult. Bear in mind that recombinant cosmids are relatively large in size (approximately 40 kbp) compared to plasmids, and thus use of the same amount (weight) of cosmids in transfection as for plasmids actually equates to lower numbers of cosmid molecules.

Q3. Is the virus isolation step necessary with the full-length DNA transfer method ?

A3. Yes, virus isolation is necessary.

There is evidence to indicate that parent virus contamination is unlikely with the full-length DNA transfer method, but the potential exists for contamination by viruses with deletions. These virus clones could potentially dominate virus stocks used for experiments or large-scale preparation if they replicated at a high rate. Therefore, virus isolation is required to eliminate these unwanted viruses at early stages of virus preparation. This step requires approximately 3 weeks, but should be considered indispensable in order to obtain reliable results (see section VI. < Importance of virus isolation >).

Q4. No recombinant viruses appear. Why is it ?

A4. Try to do a control experiment using a control cosmid pAxCAiLacZit. If recombinant viruses appear in the control experiment, the purity of the target cosmid may have been low. The purity of the cosmid has a great effect on the efficiency of transfection. If no viruses appear even in the control experiment, there may be an error in transfection. Examine the conditions of transfection. Also, try to use healthy 293 cells with the passage number as low as possible. The efficiency of infection and propagation of adenovirus may be reduced when 293 cells are subcultured for 2 months or longer.

**Reference :** When the control cosmid pAxCAiLacZit is used with the full-length DNA transfer method, cytopathic effects will be detected in 10 to 20 wells of a 10-fold dilution plate, most of which are likely to contain target viruses and can be used to prepare primary viruses. With the COS-TPC method, cytopathic effects will be detected in 10 to 20 wells of a 100-fold dilution plate, more than 50 % of which are likely to contain target viruses. The number of wells containing cells showing cytopathic effects (reflective of the number of primary virus clones obtained) may vary depending on transfection efficiency and condition of the 293 cells.

Q5. Why are the numbers of primary viruses obtained so low in comparison with the controls ?

A5. The likely causes are effects of the inserted genes.

As a consequence of these effects, the recombinant viruses obtained may differ from the target viruses, e.g., contain mutations; be sure to perform structural verification (see section VII. B-2 Confirmation of the recombinant adenovirus).

Q6. Why was cell degeneration observed in all wells ?

A6. The Adenovirus Expression Vector Kit (Dual Version) is designed so that cytopathic effects will be visible (enabling users to obtain primary viruses) in 10 to 20 wells of a 10-fold dilution plate with the full-length DNA transfer method and of a 100-fold dilution plate with the COS-TPC method (when using recombinant cosmids, such as the control cosmid pAxCAiLacZit, to which a marker gene is cloned). However, the 293 cells that die on the 18th day depending on their condition, make it difficult to be distinguished from cell degeneration caused by cytopathic effects of viruses. To lessen the potential for this to occur, be sure to use 293 cells that are at low passages and healthy (see Appendix 1). There are also cases in which cytopathic effects may be observed in nearly all wells due to factors associated with the recombinant cosmid used for transfection, changes in the amount of DNA-TPC used, the transfection methods and conditions, etc.

In such cases, because virus stocks may contain multiple clones; it is recommended that users repeat from the beginning of the procedure or clone by limiting dilution after performing confirmation of virus structure.

**Q7. The titer of the virus does not increase. Why ?**

**A7. If 293 cells are infected with virus solution of too high concentration to increase the titer, it is possible to result in an adverse effect because of the cytotoxicity (See Appendix 4). Infecting cells using MOI (multiplicity of infection) of 10 to 20 pfu/cell allow efficient adenovirus propagation in 293 cells. The titer may not increase even with the correct procedure depending on the inserted gene. In such cases, purify and concentrate the virus. (See Appendix 5 and reference 10).**

**Q8. To what degree should the virus be diluted when it is infected to cells ?**

**A8. Gene transfer using an adenovirus vector is less toxic than other methods such as electroporation and calcium phosphate coprecipitation method and allows the expression of the target gene without damaging normal functions of cells. However, when cells are infected with a virus solution of a high concentration, cells may be damaged because overproduced coat proteins of the virus may show cytotoxicity. The maximum concentration would be 10-fold dilution when high expression is necessary even at the cost of some damages to the cells and 20-fold dilution when the expression is desired to be induced without damaging normal functions of cells. However, the dilution rate varies with the target cells, and the optimal dilution must be determined according to "VII. E. Infection of target cells with the virus"**

**Q9. How long does the expression continue ?**

**A9. When cell division is not remarkable, the expression is observed from day 1, reaches a peak on day 2 or 3, is sustained for about 1 week, and decreases thereafter. However, the expression is considered to be observed partially even 3~8 weeks after. Since the adenovirus vector is present independently of the cell genome and does not replicate, it is diluted by mitosis, and the duration of the expression is shortened. Therefore, the expression is maintained at a high level by infecting the cells under the conditions that suppress cell division (e.g. after reaching full sheets), if possible.**

**Q10. What guidelines of the relevant authorities should be applied for experiments using recombinant DNA ?**

**A10. Please follow the judgment of the safety committee of your research organization and official regulations of your country.**

In any case, follow the recommendations of the safety committee of your institution.

[ In case of Japan ]

The "Ministerial ordinance concerning containment measures for category II use of genetically modified organisms in research and development etc." (Ministry of Education, Culture, Sports, Science and Technology/Ministry of the Environment No. 1, effective February 19, 2004) specifies containment measures of P2 or higher for experiments involving the use of recombinant adenoviruses. Experiments shall be performed in conformity with the containment measures specified by the ministerial ordinance. The guidelines are different in the step of preparation of the recombinant cosmid vector and the subsequent steps as shown below. Naturally, more strict regulations are applied when the target gene may have toxicity or pathogenicity.

## &lt; References &gt;

Guideline by the Ministry of Education Culture, Sports, Science and Technology.  
Ministry of the Environment (No. 1 effective February 2004.)

- Experiments to prepare recombinant cosmid vectors  
Experimental division : Class 1 or Class 2. (Class 3 depending on the DNA Donor)  
Host vector system : EK1  
Containment level : Organization approved experiments P2-P1 (P3 depending on the DNA donor)
- Experiments for generation of recombinant adenoviruses/Experiments of infection of recombinant adenoviruses  
Host : human adenovirus type5 derived nonproliferative vector.  
Experimental division : Class 2 (Class 3 depending on the DNA donor)  
Containment level : Organization approved experiments P2 (P3 depending on the DNA donor.)

Q11. Is there possibility for RCA (replication-competent adenovirus) contamination during passaging in 293 cells ?

A11. Adenovirus vector titers tend to be extremely high, so more than 5 or 6 passages are hardly required and therefore the likelihood of RCA contamination is negligible.

There is a report, however, indicating that cells passaged 10 times or more may contain some adenoviruses that have reacquired the E1A and/or E1B genes (i.e., RCA) as a result of homologous recombination with the genomic DNA of 293 cells<sup>11</sup>).

There are also numerous other reports on RCA available, such as those concerned with frequency and detection methods<sup>11, 12, 13, 14</sup>). Therefore, it is highly recommended that users test for RCA when using repeatedly passaged adenovirus vectors. A common method used for RCA screening is to infect either HeLa or A549 cells and monitor them to verify that they show no evidence of cytopathic effects; this process can be expedited by PCR method, which allows quick and highly sensitive screening (see Appendix 6).

Q12. If contamination occurs, how infective is the virus ?

A12. Since the virus does not propagate in cells other than 293 cells, it could not be infected or propagate in human, which can lead to cause symptoms of cold. Nor be transmitted from humans to humans. However, if it infects human cells at all, it is possible that the virus transports the target gene into the nucleus and expresses the gene. Therefore, make sure to perform appropriate disinfection against viruses. (In Japan, adenovirus is categorized in the P2 level. The viruses in this level range from common cold to hepatitis B virus).

Concerning adenovirus itself, wild strains of adenovirus are common, and most individuals contract cold due to type 5 adenovirus by the age of about 3 years, produce a high level of virus, and acquire neutralizing antibody.

Q13. How should disinfection be made in case of contamination (e.g. In case that the medium is spilled) ?

A13. A general disinfection method is sufficient. But people handling adenoviruses should have basic knowledge about adenoviruses as follows; Adenovirus has no envelope (derived from the cell membrane) and is encapsulated by capsid protein.

Therefore, it can be inactivated by denaturing capsid protein. All operations should be performed in a safety cabinet. For disinfection, wipe the contaminated area with 10% SDS and then with 70% ethanol. Also, adenovirus is reported to be heat labile and to be inactivated when treated at 56°C for 30 minutes. However, autoclaving should be done for 10 minutes or longer for actual disinfection. For handling adenovirus, use the tips with filter. After handling adenovirus in a safety cabinet, be sure to light the UV lamp for 30~60 minutes.

### IX. Related Products

DNA Blunting Kit (Cat.#6025)

Restriction endonuclease *Cla* I (Cat.#1034A)

Restriction endonuclease *Xho* I (Cat.#1094A)

FastPure™ DNA Kit (Cat.#9191)

### X. Note

Precautions for the use of this product

- Please follow the guideline for experiments using recombinant DNA issued by the relevant authorities and the safety committee of your organization or your country in using this product.
- The use of this product is limited for research purposes. It must not be used for clinical purposes or for *in vitro* diagnosis.
- Individual license agreement must be concluded when this product is used for industrial purposes.
- In this kit, recombinant viral particles infectious to mammalian cells are produced in 293 cells. Although this recombinant virus cannot propagate in cells other than 293 cells, in case that it attaches to the skin or the airway, it efficiently enters cells and expresses the target gene. Please use a safety cabinet to prevent inhalation or adhesion of the virus.
- Basic techniques of genetic engineering and cell cultivation are needed for the use of this product.
- The user is strongly advised not to generate recombinant adenovirus capable of expressing known oncogenes and any genes known to be hazardous to the mammals.
- Takara is not liable for any accident or damage caused by the use of this product.

**XI. Appendix****Appendix 1. 293 cells**

293 cells can be purchased from various sources, such as ATCC. Cells should be purchased at the lowest possible passage number and stocks should be prepared at low passage numbers.

Culture conditions for 293 cells are critical factors in recombinant adenovirus preparation, and cells must be maintained in optimum condition. The following section details the culture conditions used at TaKaRa Bio.

**1. Recovery of cryopreserved cells**

Remove a vial containing cryopreserved cells from a liquid nitrogen tank and place in a 37°C water bath. When nearly half-thawed, remove the vial from the water bath and allow to thaw completely with residual heat. Place cells in cell culture flasks or plates containing fresh medium at a density of approximately  $5 \times 10^4$  cells/cm<sup>2</sup> (recovery may prove difficult if the density is too low or too high).

Culture the cells in a CO<sub>2</sub> incubator (37°C, 5% ) and split when confluent.

**2. Subculture of cells**

Generally, cell culture are split every 2 to 3 days.

Split cell culture at a ratio of 1 : 2 to 1 : 4 in nearly confluent culture. Aspirate the medium and wash with PBS (–) once (do this carefully, since 293 cells detach readily.)

Aspirate the PBS (–), add 0.02% EDTA-PBS (about 1.0 ml in each T-75 flask), and incubate for 1 to 5 minutes at room temperature (if the cells do not detach readily, incubate at 37°C in a CO<sub>2</sub> incubator) to allow the cells to detach completely. Add 5 to 10 ml of culture medium and pipet up and down gently to resuspend the cells uniformly. Place an appropriate volume of cell suspension in culture flasks or plates containing fresh medium, swirl gently to distribute the suspension evenly, and culture at 37°C in a 5% CO<sub>2</sub> incubator.

**3. Culture conditions**

Culture 293 cells under the following conditions when using the Adenovirus Expression Vector Kit (Dual Version).

Culture medium : 10% FCS-DMEM

DMEM (low glucose : 1 g/L)	500 ml
Inactivated FCS	50 ml
L-Glutamine (200 mM)	5 ml
Penicillin-Streptomycin *	5 ml

Infection medium : 5% FCS-DMEM

DMEM (low glucose : 1 g/L)	500 ml
Inactivated FCS	25 ml
L-Glutamine (200 mM)	5 ml
Penicillin-Streptomycin *	5 ml

\* Penicillin : 10,000 U

\* Streptomycin : 10,000 μg/ml

Subculture : 0.02% EDTA/PBS (–)

Culture conditions : 5% CO<sub>2</sub>, 37°C

#### 4. Culturing precautions

Cells maintained in a confluent state for an extended period will become damaged and are often difficult to restore to a healthy state. On the other hand, subculture cells at too low of a density may affect their growth rate as well.

Avoid using cells passaged for 2 months or more, since cells at high passage tend to lose their uniformity, resulting in a low virus infection efficiency. The total number of passages is an important consideration as well, and therefore it is recommended to check the passage number at purchase and to prepare initial frozen stocks of the cells while they are at low passage. The passage number of cells used for experiments should ideally be 50 or lower (i.e., passage number at purchase + **passage number at laboratory**).

All of these procedures must be performed under sterile conditions, ideally in a biological safety cabinet not used to handle viruses. If the same cabinet must be used, disinfect the cabinet with ethanol and at least 1 hour exposure to UV light after each use of viruses prior to use for the above mentioned procedures.

#### 5. Storage

Recover the cells in 80% to 90% confluent, wash with medium once, resuspend in 10% DMSO/90% FCS (or commercially available cell freezing medium), and aliquot into cryotubes. Freeze the cells in a programmable freezer. If no programmable freezer is available, place the cryotubes in appropriate containers (e.g., Styrofoam boxes), freeze overnight at  $-80^{\circ}\text{C}$ , and transfer to liquid nitrogen. Cells should be cryopreserved at a density of approximately  $4 \times 10^6$  cells per tube; it is difficult to recover cells stored at too low of a density. Thaw one of the tubes in 2 to 3 days and check the condition of the cells.

It is recommended to keep the recording of the number of passages prior to freezing.

#### 6. Detection of adenovirus-infected 293 cells

In adenoviruses 10,000 viral particles are produced after infecting cells. As viruses propagate, 293 cells lose their ability to adhere to the plate and appear round and floating (Figure 5), and may be readily detached by gently tapping the side of the culture flask or plate.

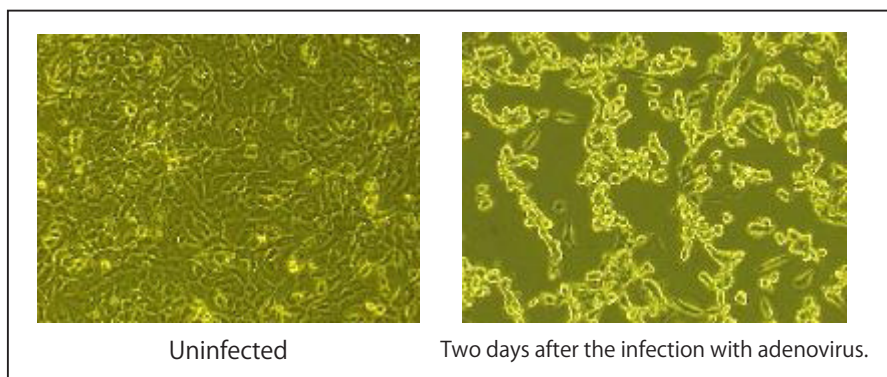


Fig.5 293 cells



**Appendix 2. Handling of recombinant adenoviruses****1. Experimental facility**

An experimental facility with a physical containment level of at least P2 is required for the handling of recombinant adenoviruses (in the step after the section "VII. B-1 Generation of the Recombinant adenovirus" described in this protocol); a facility with a higher physical containment level may be required depending on the insert gene used. Follow the DNA recombination guidelines established by the safety committee of your institution.

**2. Storage**

Highly secure screw-cap microcentrifuge tubes (e.g., tubes with O-rings) are recommended for storage of virus stocks to prevent contamination and for safer handling. Store these tubes in a deep freezer ( $-80^{\circ}\text{C}$ ) **within a P2 facility**. Do not store in a liquid nitrogen tank since tubes may burst.

**3. Freezing and thawing**

Freeze tubes containing virus stocks by tightly closing the caps and freezing quickly in powdered or crushed dry ice. Do not use liquid nitrogen for freezing since tubes may burst.

Thaw virus stock by placing tubes in a  $37^{\circ}\text{C}$  water bath. Remove the tubes when half-thawed and allow to thaw completely with residual heat. Keep thawed tubes on ice until use.

Repeating freezing and thawing may reduce titer. Store virus stocks in volumes suitable for experimental purposes in order to avoid unnecessary freezing and thawing.

**4. Recombinant virus retention period**

Adenovirus is available for long time by appropriate storage at  $-80^{\circ}\text{C}$ , because it has robust structure. However, Retesting of the titer of viruses older than 1 year after the preparation date before use is recommended.

**Appendix 3. Definition of level P2**

In Japan, the "Ministerial ordinance concerning containment measures for category II use of genetically modified organisms in research and development etc." (Ministry of Education, Culture, Sports, Science and Technology/Ministry of Environment No. 1, effective February 19, 2004) specifies containment measures of P2 or higher for experiments involving the use of recombinant adenoviruses. Experiments shall be performed in conformity with the containment measures specified by the ministerial ordinance.

The details shall be determined based on the judgment of the recombinant DNA committee at your institution.

**Appendix 4. Large-scale recombinant adenovirus preparation**

The protocol provided allows efficient large-scale recombinant virus preparation under optimum conditions.

Perform large-scale preparation of recombinant viruses according to this protocol to amplify large quantities of viruses.

A high virus titer is essential for efficient amplification. If the virus titer is unknown, first determine the titer of the virus according to with section VII. D Viral titration.

1. Prepare cultures of 293 cells that are 70 % to 100 % confluent in collagen-coated 75-cm<sup>2</sup> flasks.
  - The volumes given below are for two 75-cm<sup>2</sup> flasks; volumes may be scaled up proportionally to obtain greater quantities of virus.
2. Dilute recombinant virus stock with 5 % FCS-DMEM to prepare virus stock containing 0.5 to 1.0 × 10<sup>8</sup> pfu/ml.
3. Remove the medium from the flasks and gently add 2 ml of the virus stock prepared in step 2.
  - Infecting cells using a MOI (multiplicity of infection) of 10 to 20 pfu/cell allows efficient adenovirus propagation in 293 cells. Use of higher concentrations of virus stock for infection of 293 cells may cause cells to die and result in a lower titer.
4. Slowly swirl the flasks to disperse the virus stock solution evenly over all cells. Repeat this step 3 to 4 times every 15 to 20 minutes. The cells should be kept in a CO<sub>2</sub> incubator (37°C, 5% CO<sub>2</sub>) during this procedure.
5. Infect for 1 hour and add 13 ml of 5% FCS-DMEM.
6. After all the cells show cytopathic effects (3~4 days), collect cells with medium into sterile tubes under sterile conditions and disrupt the cells by freeze-thaw cycling or using a sealed type sonicator to liberate the viruses.
7. Collect the supernatant by centrifugation at 3000 rpm for 10 minutes at 4°C.
8. Dispense an appropriate amount into screw-cap microcentrifuge tubes, freeze quickly in dry ice, and store at -80°C.
9. Thaw one of the tubes to determine the titer (section VII. D) prior to use.
  - As described in section VII. C, it is recommended that structural confirmation, as described in section VII. B-2, and RCA screening of virus stocks are performed prior to use.

### Appendix 5. Recombinant adenoviruses purification

The use of purified recombinant virus is recommended for administration studies in animals (i.e., *in vivo* studies). The following section describes a method for adenovirus purification based on a cesium chloride step gradient method based on the reference<sup>10</sup>.

#### Equipment and reagents required

The following equipment and reagents are required for purification of recombinant adenoviruses.

- Ultracentrifuge
- Ultracentrifuge rotors
  - Swing-out rotor with a maximum speed of 28 krpm
  - Swing-out rotor with a maximum speed of 40 krpm
- Ultracentrifuge tubes
  - Ultracentrifuge tubes compatible with each rotor
- Sterilized dialysis cassette (e.g., Slide-A-Lyzer Dialysis Cassette (Thermo Fisher Scientific Inc.)) or sterilized dialysis tube (10 kD)
- 4.0 M cesium chloride/10 mM HEPES (pH7.4)
- 2.2 M cesium chloride/10 mM HEPES (pH7.4)
- Saturated cesium chloride/10 mM HEPES (pH7.4)
- 10% glycerol-PBS (-)

The pH of these reagents should be ascertained prior to use after autoclaving, since drastic changes in pH can cause infectious virus particles to lose their infectivity. These procedures must be performed under sterile conditions.

The following protocol will produce 4 to 5 ml of purified recombinant virus with a viral titer of  $10^{10}$  to  $10^{11}$  pfu/ml from recombinant viruses with titers of  $10^9$  pfu/ml or higher in quaternary virus stocks.

1. Prepare cultures of 293 cells that are 70% to 100% confluent in 6 collagen-coated 225 cm<sup>2</sup> flasks.
2. Dilute recombinant virus stock with 5 % FCS-DMEM to prepare virus stock containing  $0.6$  to  $1.2 \times 10^8$  pfu/ml.
3. Remove the medium from the flasks and gently add 5 ml per flask of the virus stock prepared in step 2.
  - Infecting cells using a MOI (multiplicity of infection) of 10 to 20 pfu/cell allows efficient adenovirus propagation in 293 cells. Use of higher concentrations of virus stock for infection of 293 cells may cause cells to die and result in a lower titer.
4. Slowly swirl the flasks to disperse the virus stock solution evenly over all cells. Perform this step 3 to 4 times every 15 to 20 minutes. The cells should be kept in a CO<sub>2</sub> incubator (37°C, 5% CO<sub>2</sub>) during this time.
5. Infect for 1 hour and add 30 ml of 5% FCS-DMEM per flask (35 ml in total).
6. After all the cells show cytopathic effects (3~4 days), transfer the cells with medium into 6 sterile tubes and centrifuge at 3,000 rpm for 10 minutes at 4°C.
7. Discard the supernatant leaving 10 ml and disrupt the cells using a sealed type sonicator to liberate the viruses.
  - The use of a sealed type sonicator is recommended. Do not use open sonicators as they generate aerosols.
8. Collect the supernatant by centrifugation at 3000 rpm for 10 minutes at 4°C.
  - These "concentrated" virus stocks have strong cytotoxicity and therefore must be purified before use (see section VIII. Q&A. Q7).
9. Perform the first cesium chloride density gradient-based virus purification by layering the following reagents in the order shown in a tube compatible with a swing-out rotor with a maximum speed of 28 krpm.
 

4.0 M cesium chloride/10 mM HEPES	10 ml
2.2 M cesium chloride/10 mM HEPES	5 ml
Virus stock	20 ml

  - Caution : Tubes filled with liquid to less than 0.5 to 1 mm from the top may break during ultracentrifugation.
10. Centrifuge at 25,000 rpm for 2 hours at 4°C using a swing-out rotor.
11. Collect the virus band using a capillary tube etc.
  - The virus band will be readily visible against a black background. The upper white band consists of cellular proteins. Two to 3 ml of virus stock can be recovered from 20 ml of the virus stock obtained in step 8.
12. Add an equal volume of saturated cesium chloride to the recovered virus stock obtained in step 11.
13. Perform the second cesium chloride density gradient-based virus purification by layering the following reagents in the order shown in a tube compatible with a swing-out rotor with a maximum speed of 40 krpm.
 

Recovered virus + saturated cesium chloride	4 to 5 ml
4.0 M cesium chloride/10 mM HEPES	2 ml
2.2 M cesium chloride/10 mM HEPES	3 to 4 ml

14. Centrifuge at 35,000 rpm for 3 hours at 4°C using a swing-out rotor.
15. Collect the virus band using a capillary tube etc.
  - If two bands are visible, the bottom band will be the virus band (the upper band consists of proteins).
16. Transfer to a sterilized dialysis cassette (or sterilized dialysis tube) under sterile conditions and dialyze against 1 L of 10 % glycerol in PBS (–). Replace the outer solution after 2 hours and dialyze overnight.
17. Collect the dialyzed virus stock, aliquot for each use, and store at –80°C.
18. Thaw one of the tubes and determine the titer (see section VII. D) prior to first use.
  - As described in section VII. C, it is recommended that structural confirmation, as described in section VII. B-2, and RCA screening of virus stocks are performed prior to use.

#### Appendix 6. RCA screening < PCR method >

Since the COS-TPC method uses homologous recombination for recombinant adenovirus production, there is an associated risk for the presence of RCAs (replication competent adenoviruses) with the E1 gene in virus stocks prepared by the method. Even virus samples that did not contain RCAs initially may still acquire the E1 gene during the course of multiple passages in 293 cells<sup>11, 12</sup>. It is therefore recommended to check for RCAs.

A common method used for RCA screening is to infect cells (e.g., HeLa cells or A549 cells) and verify that they show no cytopathic effects. This section introduces a PCR-based RCA screening method developed by Dr. Saito and colleagues which allows quick and highly sensitive detection.

There are a number of reports available on PCR-based RCA screening methods<sup>11, 13, 14</sup>.

#### Equipment and reagents required

PCR-based RCA screening will require the following additional equipment and reagents :

- PCR Thermal cyclers
  - Ex.
  - TaKaRa PCR Thermal Cyclers Dice
  - TaKaRa PCR Thermal Cyclers GP
- *TaKaRa Taq* (Cat.#R001A)
- dNTP mixture (included in Cat.#R001A)
- 10× PCR Buffer (included in Cat.#R001A)
- Primer sets

Ex. 1 : Primer set designed to amplify 415 bp beginning at the start codon of E1A

Sense : 5'-ATGAGACATATTATCTGCCACGGAGGTGTTATTAC-3'  
(Adenovirus type 5; 560~594 bp)

Antisense : 5'-CCTCTTCATCCTCGTCGTCCTGGGTGGAAAGCCA-3'  
(Adenovirus type 5; 974~940 bp)

Ex. 2 : Primer set designed to amplify 240 bp beginning at the start codon of E1A

Sense : 5'-ATGAGACATATTATCTGCCAC-3'  
(Adenovirus type 5; 560~580 bp)

Antisense : 5'-GTAAGTCAATCCCTTCCTGCAC-3'  
(Adenovirus type 5; 800~779 bp)

1. Prepare HeLa cells that are 80% to 90% confluent in 24-well plates.
2. Aspirate the medium.
3. Add 10  $\mu$ l of recombinant virus sample (1  $\mu$ l if purified viruses are used) and 0.1 ml of 5% FCS-DMEM.
4. Slowly swirl the flasks to distribute the virus stock solution evenly over all cells. Perform this step 3 to 4 times every 15 to 20 minutes. The cells should be kept in a CO<sub>2</sub> incubator (37°C, 5% CO<sub>2</sub>) during this time.
5. Infect for 1 hour and add 0.4 ml of 5% FCS-DMEM.
6. Culture for 3 days.
  - After 3 days, verify microscopically that no cytopathic effects are visible.
7. Aspirate the medium, detach the cells from the plate using trypsin, and centrifuge. Wash the cell ppt with 1 ml of PBS 3 times.  
To screen for RCAs, extract the whole DNA from HeLa cells using the following method and perform PCR to detect the E1 gene. It is possible to easily, rapidly extract DNA by using FastPure™ DNA Kit in plate of Step 8~13 below.
  - Sufficient washing of cells in this step is essential. If the washing in this step is insufficient when using unpurified virus samples, the E1 gene DNA derived from the 293 cells contained in the virus sample solution can not be eliminated completely. This is difficult to do assessment of a result obtained.
8. Aspirate the PBS and add the following reagents to HeLa cells to a final volume of 400  $\mu$ l.

10×TNE Buffer	40 $\mu$ l
Proteinase K (20 mg/ml)	4 $\mu$ l
Sterile distilled water	up to 400 $\mu$ l

9. Collect mixture into sterile tubes and suspend by vortexing.
10. Add 4  $\mu$ l of 10% SDS and mix well by vortexing.
11. Incubate at 50°C for 1 hour.
12. Extract with phenol/chloroform twice and with chloroform twice.
  - Mix well by vortexing.
13. Precipitate with ethanol and dissolve the DNA pellet in 50  $\mu$ l of TE Buffer containing 20  $\mu$ g/ml RNase A.
  - It may be difficult to dissolve the DNA pellet if allowed to dry completely following ethanol precipitation.
14. Prepare the PCR mixture as follow :

DNA sample *	2 $\mu$ l
TAKARA Taq (5 U/ $\mu$ l)	0.5 $\mu$ l
dNTP mix (2.5 mM/dNTP)	4 $\mu$ l
10×PCR buffer (Mg <sup>2+</sup> plus)	5 $\mu$ l
Sense primer (20 pmol/ $\mu$ l)	0.5 $\mu$ l
Antisense primer (20 pmol/ $\mu$ l)	0.5 $\mu$ l
dH <sub>2</sub> O	
<hr/> Total :	50 $\mu$ l

\* : DNA sample : DNA solution prepared in step 13.

- \* : Negative control
    - Reaction mixture containing 2  $\mu$ l of sterile distilled water in place of a DNA sample
    - Reaction mixture containing 2  $\mu$ l of DNA sample extracted from uninfected HeLa cells in the same manner as described in steps 7 to 13.
  - \* : Positive control
    - Reaction mixture containing 2  $\mu$ l of DNA sample extracted from uninfected 293 cells in the same manner as described in steps 7 to 13.
15. Carry out the reaction using the following conditions.
- |      |         |  |
|------|---------|--|
| 94°C | 30 sec. | } 25 cycles (primer pair in Ex. 1)<br>or<br>30 cycles (primer pair in Ex. 2) |
| 55°C | 30 sec. |  |
| 72°C | 30 sec. |  |
- Do not run more than 30 cycles, as this increases the background and makes detection difficult.
16. Run 7  $\mu$ l of PCR product on agarose gel and confirm the size of the amplified product.
- The presence of corresponding bands suggests a high probability of RCA contamination.



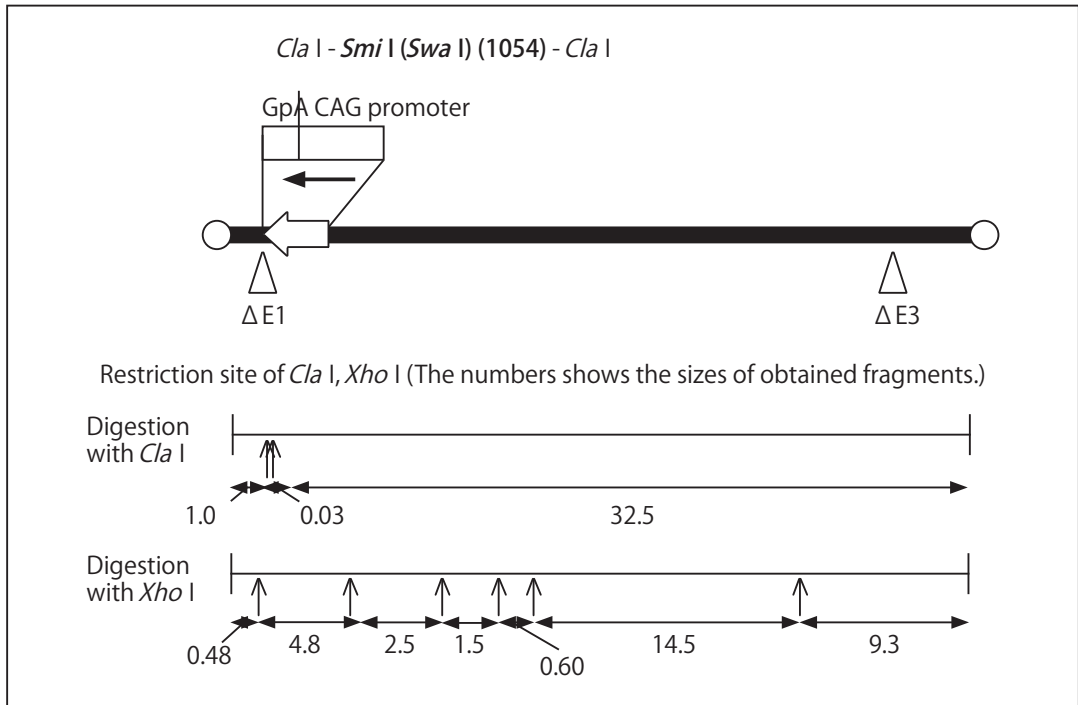


Fig. 6-3 Structure of recombinant adenovirus AxCawt2.

Constructed based on a cosmid vector AxCawt2 not to include an insert gene.

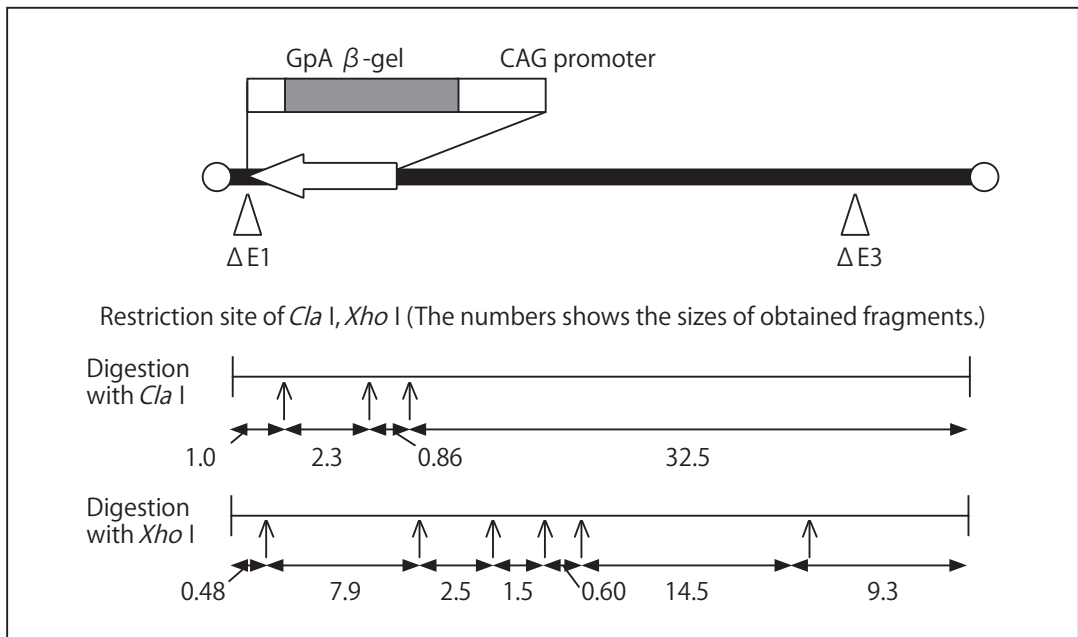


Fig. 6-4 Structure of recombinant adenovirus AxCaiLacZ.

Constructed based on a recombinant cosmid pAxCaiLacZit to have  $\beta$ -galactosidase gene.



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**NOTICE TO PURCHASER: LIMITED LICENSE****[L28] Adenovirus**

This product is covered by the claims of U.S. Patent No. 5,731,172 and its foreign counterpart patent claims.

**[L30] Adenovirus CAG promoter**

This product is covered by the claims of Japanese Patent No. 2,824,434 and its foreign counterpart patent claims.

**[L31] Adenovirus Dual Cosmid**

This product is the subject of the pending U.S. patent application and its foreign counterparts.

**NOTE :** This product is intended to be used for research purpose only. They are not to be used for drug or diagnostic purposes, nor are they intended for human use. They shall not to be used products as food, cosmetics, or utensils, etc.

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