InterPlay Adenoviral TAP System

Instruction Manual

Catalog #240213 InterPlay Adenoviral N-terminal TAP System
#240215 InterPlay Adenoviral C-terminal TAP System
#240214 InterPlay Adenoviral N-terminal TAP Vectors
#240216 InterPlay Adenoviral C-terminal TAP Vectors

Revision C.0

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240213-12

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InterPlay Adenoviral TAP System

CONTENTS

InterPlay Adenoviral TAP System*

MATERIALS PROVIDED

Materials provided	Catalog #240213	Catalog #240215	Catalog #240214	Catalog #240216
InterPlay Adenoviral shuttle vectors				
pNTAP Shuttle vector-A (1 µg/µl in TE buffer)	20 µg	—	20 µg	—
pNTAP Shuttle vector-B (1 µg/µl in TE buffer)	20 µg	—	20 µg	—
pNTAP Shuttle vector-C (1 µg/µl in TE buffer)	20 µg	—	20 µg	—
pCTAP Shuttle vector-A (1 µg/µl in TE buffer)	—	20 µg	—	20 µg
pCTAP Shuttle vector-B (1 µg/µl in TE buffer)	—	20 µg	—	20 µg
pCTAP Shuttle vector-C (1 µg/µl in TE buffer)	—	20 µg	—	20 µg
pTAP Shuttle-CAT control vector (1 µg/µl in TE buffer)	20 µg	20 µg	20 µg	20 µg
Adenoviral cloning components				
pShuttle-CMV-*lacZ* control vector (Catalog #240008; 1 µg/µl in TE buffer)	10 µg	10 µg	10 µg	10 µg
AD-293 cells (Catalog #240085; provided in 1 ml of DMEM + 40% FBS + 10% DMSO)	1×10^6 cells	1×10^6 cells	—	—
BJ5183-AD-1 electroporation competent cells (Catalog #200157)[a]	5 × 100 µl	5 × 100 µl	—	—
Transformation control plasmid (for BJ5183-AD-1 electroporation competent cells; 0.1 ng/µl in TE buffer)	10 µl	10 µl	—	—
XL10-Gold ultracompetent cells (Catalog #200314)[b]	5 × 100 µl	5 × 100 µl	—	—
XL10-Gold β-mercaptoethanol mix (β-ME)	50 µl	50 µl	—	—
pUC18 DNA control plasmid (for XL10-Gold ultracompetent cells; 0.1 ng/µl in TE buffer)	10 µl	10 µl	—	—
InterPlay TAP purification kit (#240107)[c]				
Lysis buffer	50 ml	50 ml	—	—
0.5 M EDTA	200 µl	200 µl	—	—
14.4 M β-mercaptoethanol	69 µl	69 µl	—	—
Streptavidin resin (#240105)[d]	1.25 ml	1.25 ml	—	—
Streptavidin binding buffer (SBB)	25 ml	25 ml	—	—
Streptavidin elution buffer (SEB)	5 ml	5 ml	—	—
Streptavidin supernatant supplement	100 µl	100 µl	—	—
MS-Grade calmodulin resin (#240106)[d]	0.625 ml	0.625 ml	—	—
Calmodulin binding buffer (CBB)	2 × 20 ml	2 × 20 ml	—	—
Calmodulin elution buffer (CEB)	2.5 ml	2.5 ml	—	—

[a] Each 100 µl aliquot is sufficient for two transformations.

[b] Each 100 µl aliquot is sufficient for one transformation.

[c] The InterPlay TAP purification kit provides reagents sufficient for 5 purifications (1×10^8 cells/purification).

[d] The resin volume listed applies to the settled resin only. The resin is supplied as a 50% slurry, bringing the total volume to twice the amount listed.

Revision C.0

* Patent Pending

STORAGE CONDITIONS

All Vectors: –20°C

Competent Cells (including β-ME mix): Place at –80°C immediately upon arrival. Do not place competent cells in liquid nitrogen.

AD-293 Cells: Place in liquid nitrogen immediately upon arrival.

All other components: Store at 4°C upon receipt. Do not freeze.

ADDITIONAL MATERIALS REQUIRED

1× PBS, sterile[§]
14-ml BD Falcon polypropylene round-bottom tubes (BD Biosciences Catalog #352059)
5-ml BD Falcon polystyrene round bottom tubes (BD Biosciences Catalog #352054)
Anti-Calmodulin binding protein epitope tag antibody (Upstate Catalog #07-482)
Electroporation cuvettes, 0.2 cm gap
Electroporator
GeneJammer transfection reagent (Agilent Catalog #204130)
Growth medium for AD-293 cells[§] (Invitrogen Life Technologies [Gibco] Catalog #11995)
In Situ β-Galactosidase staining kit (Agilent Catalog #200384)
Media for cell growth and transfection
Microcon® YM-10 centrifugal filter unit (Millipore Catalog #42421)
Pac I restriction enzyme
PEG compound (Sigma Catalog #P2263)
Pme I restriction enzyme
Protease inhibitors (e.g., Protease inhibitor cocktail [Sigma Catalog #P8340], PMSF [Sigma Catalog #P7626], etc.)
SeaPlaque® agarose (FMC Corporation)
StrataPrep PCR purification kit (Agilent Catalog #400771)

[§] See *Preparation of Media and Reagents.*

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INTRODUCTION

Identification of protein–protein interactions is at the core of understanding biological processes occurring in living cells. Traditionally, potential interacting proteins have been identified by genetic methods (two–hybrid screens) with subsequent verification of the interaction by co-immunoprecipitation. While this method has been successful for detection of two interacting proteins, it is of limited utility when more complex protein aggregates such as ribosomes, spliceosome complexes, or transcription complexes are investigated. To overcome this limitation, an alternative method was developed for purification of yeast protein complexes.[1,2] This tandem affinity purification (TAP) method combines purification of a protein complex of interest using affinity purification tags with subsequent mass spectrometry identification of unknown protein complex components. The key feature of this technology is the use of two different affinity purification tags that are fused to at least one known component of the protein complex of interest by genetic methods. Performing two consecutive purification steps using affinity purification tags that have gentle washing and small molecule elution conditions allows for isolation of exceptionally clean proteins without disrupting the targeted complex.

The Agilent Interplay TAP systems improve upon the original published protocol with two peptide tags that allow for isolation of exceptionally clean proteins without disrupting the targeted complex (see Figures 1 and 2). The SBP tag, a synthetic sequence isolated from a random peptide library, has a high affinity for the streptavidin resin provided (~2×10^{-9} M), and can be effectively eluted with biotin.[3,4] The CBP tag, derived from a C-terminal fragment of muscle myocin light-chain kinase, has a high affinity for the calmodulin resin provided (~1×10^{-9} M) in the presence of calcium. Upon removal of calcium with a chelating agent, recovery of the tagged protein from the resin is achieved.[5–7] Both tags can be eluted from their respective resins with gentle washing and small molecule elution conditions thus increasing the amount and purity of the resulting purified protein complex.

SBP tag	**MDEKTTGWRGGHVVEGLAGELEQLRARLEHHPQGQREPSGGCKLG**
CBP tag	**KRRWKKNFIAVSAANRFKKISSSGAL**

FIGURE 1 Amino acid sequences of the streptavidin (SBP) and calmodulin binding peptides (CBP).

Overview of the InterPlay Adenoviral TAP System

The InterPlay Adenoviral TAP System couples adenoviral delivery of your gene of interest with the SBP and CBP tandem affinity purification technology to further enhance your protein-protein interaction studies. Adenoviral vectors offer delivery of the gene of interest to an increased number of cells compared to standard transfection methods and, once viral supernatants are produced, there is greater versatility in the types of target cells that can be studied.

The InterPlay Adenoviral TAP System uses a method developed by T.C. He and colleagues[8] to generate recombinant adenovirus. A schematic overview of the production of recombinant adenovirus is shown in Figure 3. The DNA of interest is cloned into one of the two TAP shuttle vectors, each available in three different reading frames: Adenoviral pNTAP Shuttle vectors A, B, or C or Adenoviral pCTAP Shuttle vectors A, B, or C. Once constructed, the TAP shuttle vector with insert is linearized with *Pme* I and transformed into BJ5183-AD-1 competent cells, which carry the pAdEasy-1 plasmid. Transformants are selected for kanamycin resistance, and recombinants are subsequently identified by restriction digestion. Once a recombinant is identified, it is produced in bulk using the recombination-deficient XL10-Gold strain. Purified recombinant adenoviral plasmid DNA is digested with *Pac* I to expose its inverted terminal repeats (ITRs), and is then used to transfect AD-293 cells where deleted viral assembly genes are complemented in vivo. The AD-293 cells, included with the system, are HEK293-derived cells with improved cell adherence properties which simplify the process of producing recombinant adenovirus particles.

Following production of recombinant adenovirus (see Figure 3) and subsequent adenoviral transduction of the target cells, cells are harvested and the proteins are purified using streptavidin and calmodulin resins (see Figure 2). The intact purified protein complex is analyzed by SDS-PAGE and the known gene of interest is detected by Western blotting using an appropriate antibody, if available, indicating its presence and the approximate size of its co-purified, interacting partner. The proteins are further characterized by in-gel digestion with trypsin followed by mass spectrometry analysis. See Figure 4 for a comprehensive flowchart.

Advantages of Recombinant Adenovirus for Gene Expression

Broad range of infectivity and high titer

Adenoviruses can infect a broad range of mammalian cells and have been used successfully to express human and non-human proteins. Recombinant adenoviruses can produce high titers (10^7 to 10^8 pfu/ml following transient transfection).

Infection does not require an actively dividing host cell

Recombinant adenovirus can infect both dividing and non-dividing cells.

Expressed human proteins are properly folded and modified

Because the AdEasy vector system employs human adenovirus and human host cell lines, human proteins expressed using this system are abundant and have the correct posttranslational modification and folding.

Large insert size

The AdEasy vector system, when used with the pTAP shuttle vectors, allows for the insertion of up to 6.6 kb of foreign DNA.

AdEasy vector is non-insertional

Because the recombinant adenovirus remains epichromosomal in the human host cell, there is only a remote possibility of activation or inactivation of host cell genes resulting from interruption by the transfected gene(s).

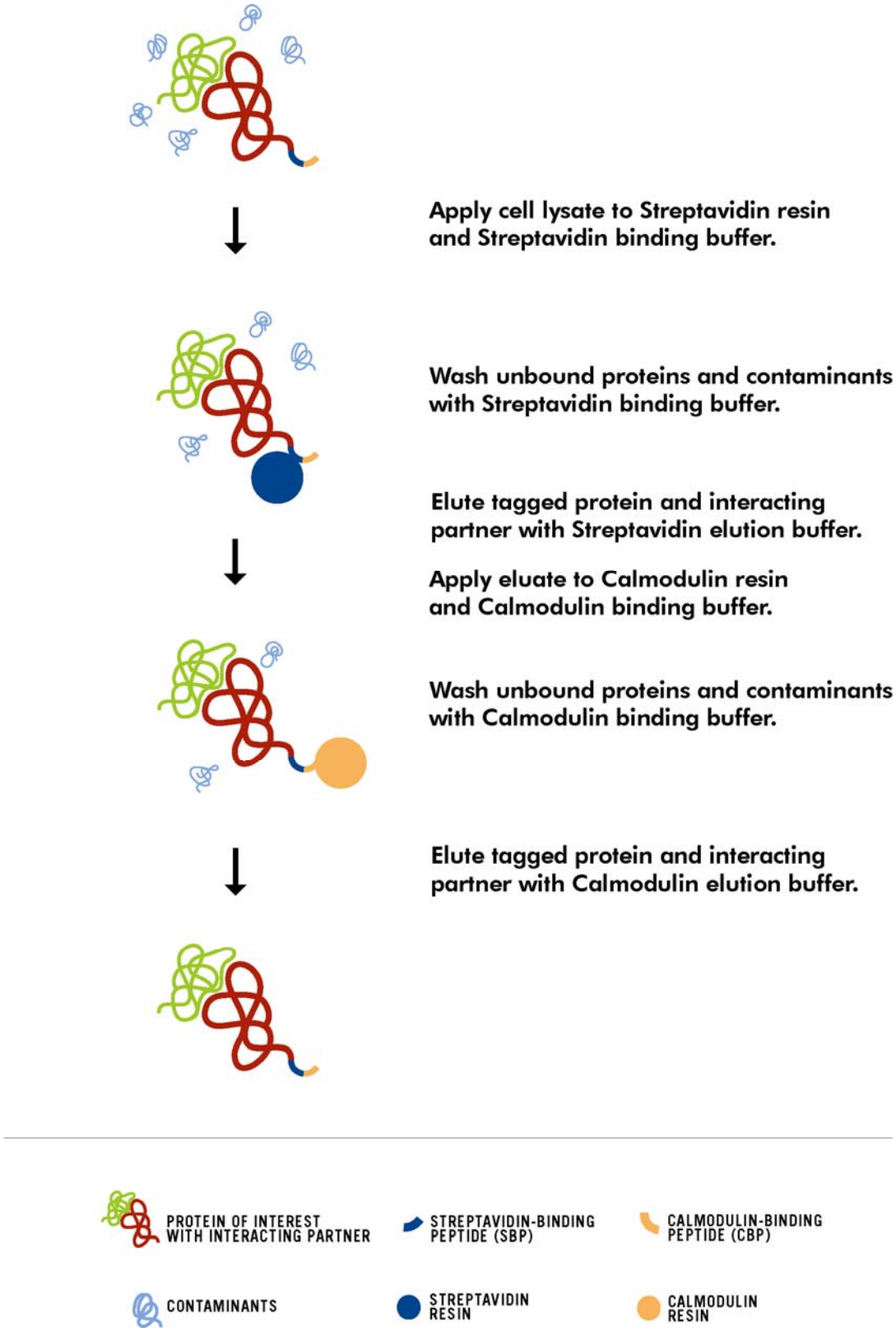


FIGURE 2 Tandem affinity purification of the tagged protein of interest and interacting proteins using streptavidin resin followed by calmodulin resin.

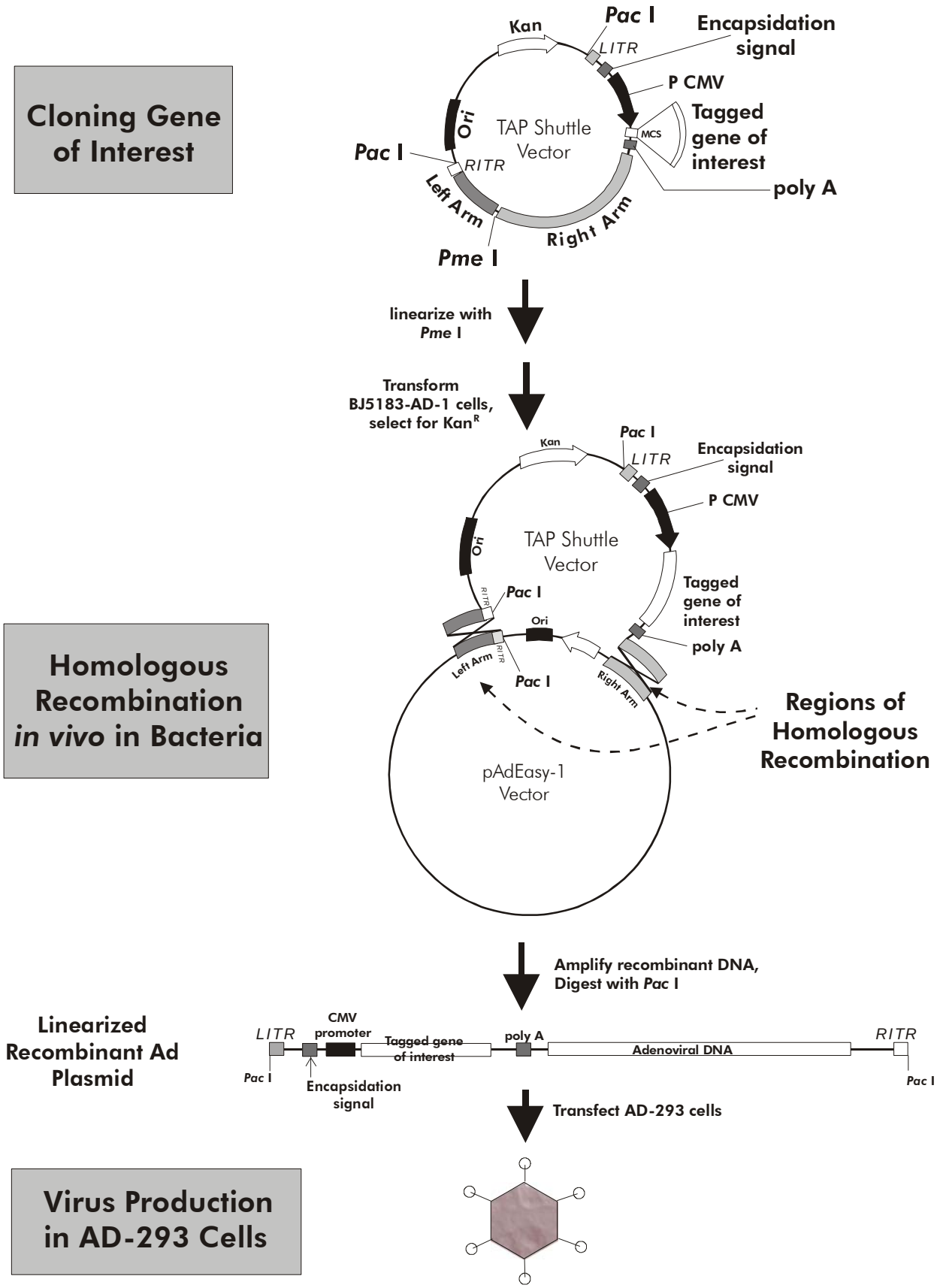


FIGURE 3 Production of recombinant adenovirus using the AdEasy™ adenoviral vector system.

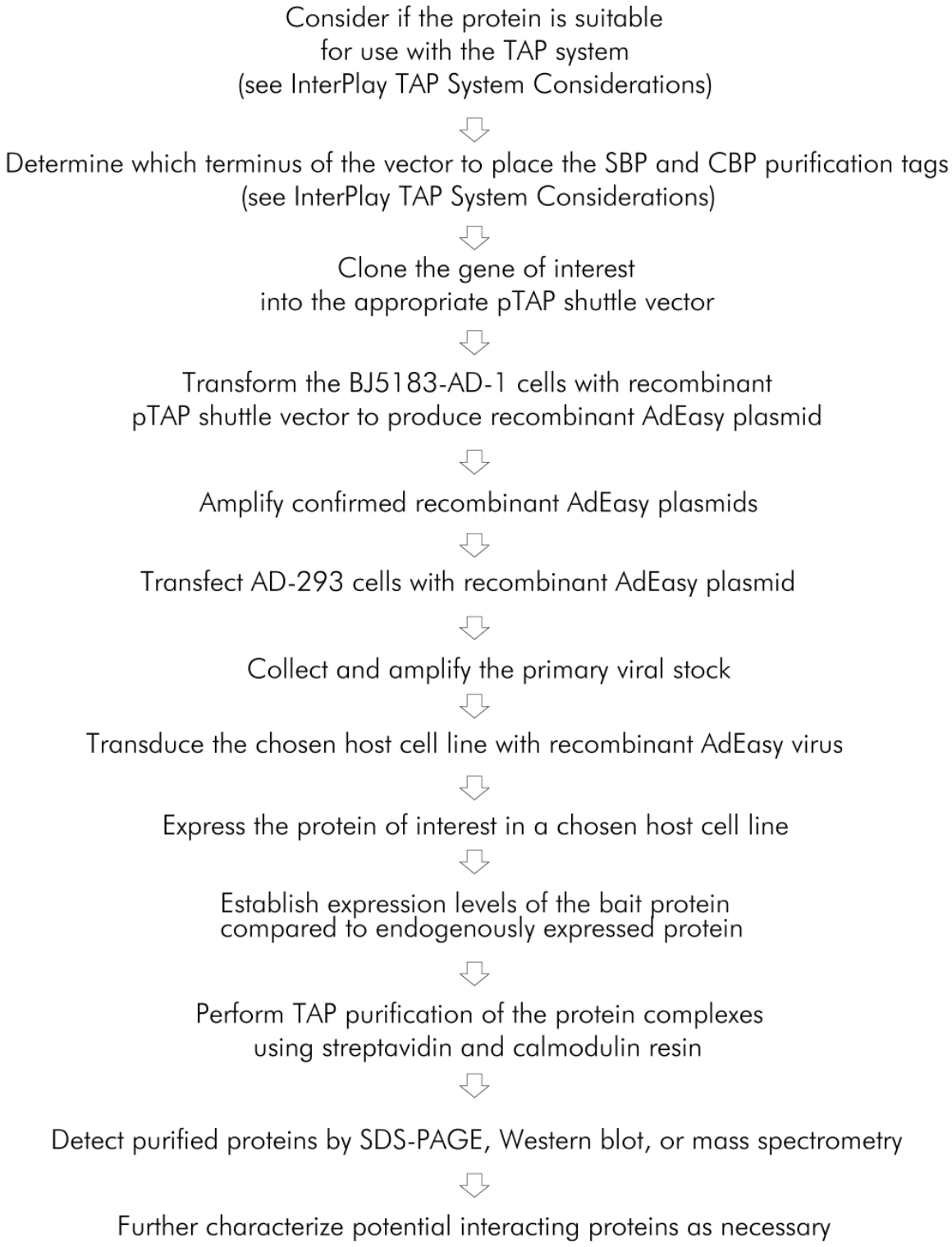


FIGURE 4 Flowchart of InterPlay Adenoviral TAP System steps.

INTERPLAY TAP SYSTEM CONSIDERATIONS

Ectopic expression of the TAP-tagged bait protein in the host cell results in competition of the TAP-tagged bait with the untagged endogenous protein. To ensure sufficient recovery of interacting proteins with the TAP-tagged bait, the TAP-tagged bait should be expressed at a level equal to or greater than the level of the endogenously expressed protein.

Although the TAP procedure results in extraordinary enrichment of the TAP-tagged bait and the associated proteins, some interactions (such as those with heat shock proteins) may be nonspecific. In order to confidently identify bait-specific interactions, performing a control purification with an unrelated bait is strongly recommended.

TAP-tagged bait proteins may behave differently depending on which terminus is fused to the TAP tag. Ideally, the best terminus for placement of the SBP and CBP tandem tags should be determined empirically. However, structure databases can provide valuable information about the accessibility of the respective termini (e.g., the NCBI structure database; *http://www.ncbi.nlm.nih.gov/Structure*). In general, tag placement at the N-terminus of the protein results in higher expression than placement at the C-terminus.

If the bait protein forms homodimers, there will be a tendency to preferentially purify tagged bait homodimers, thus potentially interfering with the purification of heterodimerizing target proteins. Certain modifications of the bait protein (mutations or truncations) may eliminate homodimerization, but this approach may also eliminate the formation of heterodimers.

DESCRIPTION OF THE VECTORS

pAdEasy-1 Vector

The pAdEasy-1 vector contains the ampicillin resistance gene and the pBR322 origin of replication.

The pAdEasy-1 vector, containing most of the human adenovirus serotype 5 (Ad5) genome, is deleted for the genes E1 and E3. The removal of these two viral genes creates space for foreign DNA and eliminates self-replication capabilities.[9] The E1 deletion renders the viruses defective for replication and incapable of producing infectious viral particles in target cells (provided there is no complementation by the host cell); the E3 region encodes proteins involved in evading host immunity and is dispensable. The deletion of both genes creates room for up to 7.5 kb of foreign DNA that can be inserted into the Ad5 genome. The E1 gene, which is necessary for production of viral particles, is provided in *trans* by AD-293 cells. pAdEasy-1 carries the ampicillin resistance gene, which is lost after recombination with a shuttle vector. (See Figure 5.)

pNTAP Shuttle and pCTAP Shuttle Vectors

In each of the adenoviral TAP shuttle vectors, eukaryotic expression is driven by the human cytomegalovirus (CMV) immediate early promoter which directs constitutive expression of cloned inserts in a wide variety of mammalian cell lines. Each vector contains the kanamycin-resistance gene under control of the prokaryotic β-lactamase promoter to provide kanamycin resistance in bacteria.

The pNTAP shuttle and pCTAP shuttle vectors are used to clone a gene of interest with the SBP and CBP affinity tags adjacent to the 5´ end and 3´ end of the gene, respectively. The vector contains a multiple cloning site that is suitable for insertion of a large cDNA (up to 6.6 kb). The regions indicated as arms are the stretches of sequence homology with pAdEasy-1 where the homologous recombination occurs. The R-ITR and L-ITR regions are short inverted terminal repeats (Left and Right) which have a role in replication of the viral DNA.[10] The pNTAP shuttle and pCTAP shuttle vectors are available in three different reading frames to simplify subcloning. These reading frames, designated as A, B, and C, differ only by one or two bases. Thus, each shuttle vector has a reading frame that will allow cloning a gene of interest so that it is fused correctly with the affinity tags. The multiple cloning site (MCS) allows for a variety of cloning strategies, resulting in fusion of the TAP tags to the N- or C-terminus of the protein of interest. (See Figures 6 and 7.)

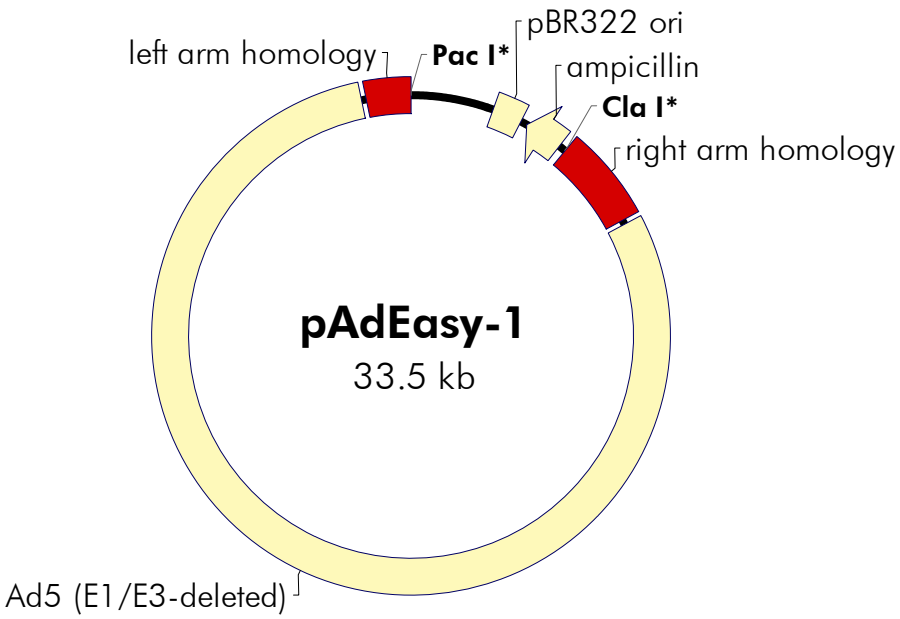
pTAP Shuttle-CAT Control Vector

The pTAP Shuttle-CAT control vector contains the chloramphenicol acetyltransferase (CAT) ORF tagged at the N-terminus with SBP and CBP tags (see Figure 8). The recombinant pTAP Shuttle-CAT adenovirus can be used to generate a set of background proteins for mass spectrometry analysis produced by the TAP purification procedure.

pShuttle-CMV-lacZ Control Vector

The *lacZ* gene was inserted in the MCS site of the pShuttle-CMV to produce pShuttle-CMV-*lacZ* (see Figure 8). This construct is provided as a control for the production of recombinant adenovirus.

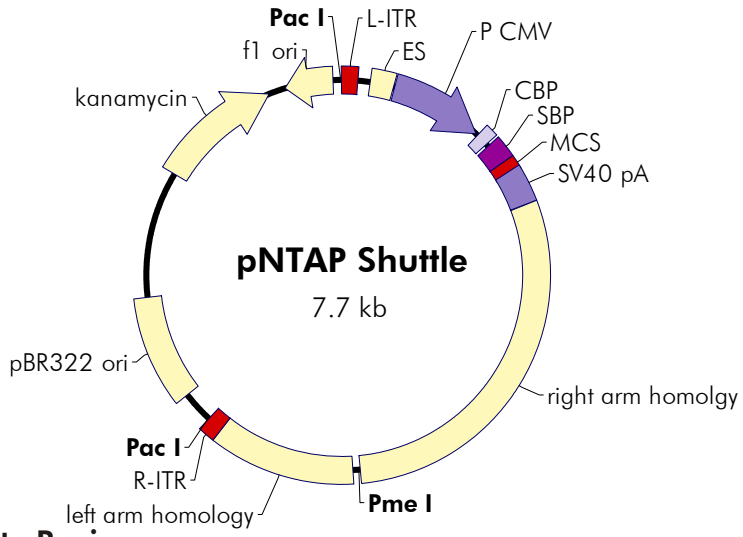
pAdEasy-1 Vector Map



Feature	Nucleotide Position
pBR322 origin of replication	1854–2521
ampicillin resistance (*bla*) ORF	2669–3529
Ad5 right arm homology	3716–5721
Ad5 left arm homology	32483–33471

FIGURE 5 The pAdEasy-1 vector

pNTAP Shuttle Vector Map



pNTAP-Shuttle Multiple Cloning Site Region
(sequence shown 1156–1297)

SBP tag (3´ end)

P S G G C K L G

Srf I | Sal I

CCCTCCGGCGGCTGCAAGCTGGGC * GCCCGGGCGGATCCCCCGGGCGTCGAC...

Not I | Xho I | Hind III | Cla I | Mlu I | Pvu I | EcoR V

...GCGGCCGCTCGAGCCGAAGCTTATCGATACGCGTCCGATCGGATATCCGATCCACCGGA...

Xba I

Reverse primer binding site

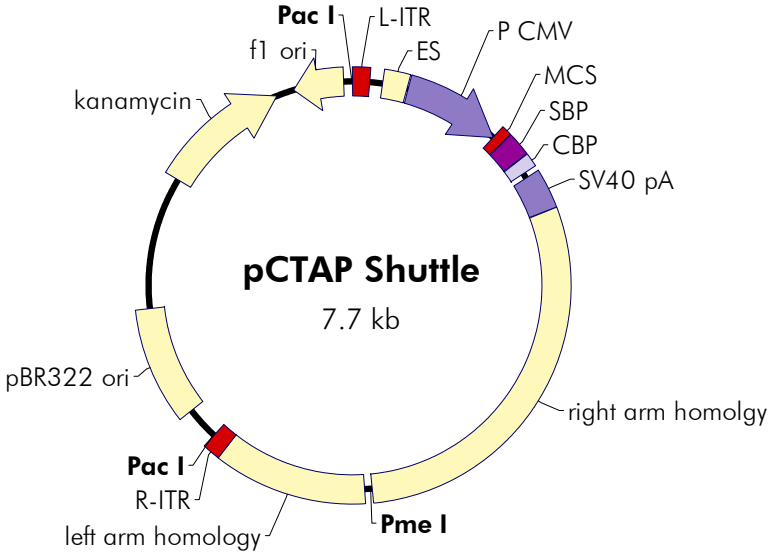
...TCTAGATAACTGATCATAATCAGCCATACCAC

* In pNTAP-A, no bases inserted; in pNTAP-B, T inserted; in pNTAP-C, TT inserted

Feature	Nucleotide Position
left Ad5 inverted terminal repeat (ITR)	1–103
encapsidation signal (ES)	183–331
CMV promoter	341–933
forward primer binding site	888–907
calmodulin binding peptide (CBP)	949–1026
streptavidin binding peptide (SBP)	1045–1179
multiple cloning site	1180–1271
reverse primer binding site	1275–1297
SV40 polyA signal	1277–1504
Ad5 right arm homology	1509–3763
Ad5 left arm homology	3811–4694
right Ad5 inverted terminal repeat (ITR)	4695–4797
pBR322 origin of replication	5001–5668
kanamycin resistance ORF	6477–7268
f1 (–) origin of ss-DNA replication	7681–7376

FIGURE 6 Circular map of the pNTAP Shuttle A–C expression vectors. The positions listed in the table above correspond to pNTAP Shuttle-A. See Table I for a complete list of feature positions for the pNTAP Shuttle-A–C vectors.

pCTAP Shuttle Vector Map



pCTAP-Shuttle Multiple Cloning Site Region
(sequence shown 888–1034)

Forward primer binding site

GGTCTATATAAGCAGAGCTGGTTTAGTGAACCGTCAGATCCGCTAG...

Bgl II Kpn I Sal I Not I Xho I Hind III Cla I Mlu I Pvu I

...AGATCTGGTACCGTCGACGCGGCCGCTCGAGCCTAAGCTTATCGATACGCGTCCGATCG*...

SBP tag (5′ end)

M D E K T T G W R G G H

...GCAGCAATGGACGAGAAGACCACCGGCTGGCGGGGCGGCCAC

* In pCTAP-A, no bases inserted; in pCTAP-B, A inserted; in pCTAP-C, GA inserted

Feature	Nucleotide Position
left Ad5 inverted terminal repeat (ITR)	1–103
encapsidation signal (ES)	183–331
CMV promoter	341–933
forward primer binding site	888–907
multiple cloning site	934–992
streptavidin binding peptide (SBP)	999–1133
calmodulin binding peptide (CBP)	1140–1217
reverse primer binding site	1248–1270
SV40 polyA signal	1250–1477
Ad5 right arm homology	1482–3736
Ad5 left arm homology	3784–4667
right Ad5 inverted terminal repeat (ITR)	4668–4770
pBR322 origin of replication	4974–5641
kanamycin resistance ORF	6450–7241
f1 (–) origin of ss-DNA replication	7654–7349

FIGURE 7 Circular map of the pCTAP Shuttle A–C expression vectors. The positions listed in the table above correspond to pCTAP Shuttle-A. See Table I for a complete list of feature positions for the pCTAP Shuttle-A–C vectors.

TABLE I

Locations of Features for the pNTAP Shuttle-A–C and pCTAP Shuttle-A–C Vectors

Feature	pNTAP Shuttle-A	pNTAP Shuttle-B	pNTAP Shuttle-C	pCTAP Shuttle-A	pCTAP Shuttle-B	pCTAP Shuttle-C
L-ITR	1–103	1–103	1–103	1–103	1–103	1–103
ES	183–331	183–331	183–331	183–331	183–331	183–331
P CMV	341–933	341–933	341–933	341–933	341–933	341–933
CBP	949–1026	949–1026	949–1026	1140–1217	1141–1218	1142–1219
SBP	1045–1179	1045–1179	1045–1179	999–1133	1000–1134	1001–1135
MCS	1180–1271	1181–1272	1182–1273	934–992	934–992	934–992
SV40 pA	1277–1504	1278–1505	1279–1506	1250–1477	1251–1478	1252–1479
right arm	1509–3763	1510–3764	1511–3765	1482–3736	1483–3737	1484–3738
left arm	3811–4694	3812–4695	3813–4696	3784–4667	3785–4668	3786–4669
R-ITR	4695–4797	4696–4798	4697–4799	4668–4770	4669–4771	4670–4772
pBR322 ori	5001–5668	5002–5669	5003–5670	4974–5641	4975–5642	4976–5643
kan	6477–7268	6478–7269	6479–7270	6450–7241	6451–7242	6452–7243
f1 origin	7681–7376	7682–7377	7683–7378	7654–7349	7655–7350	7656–7351

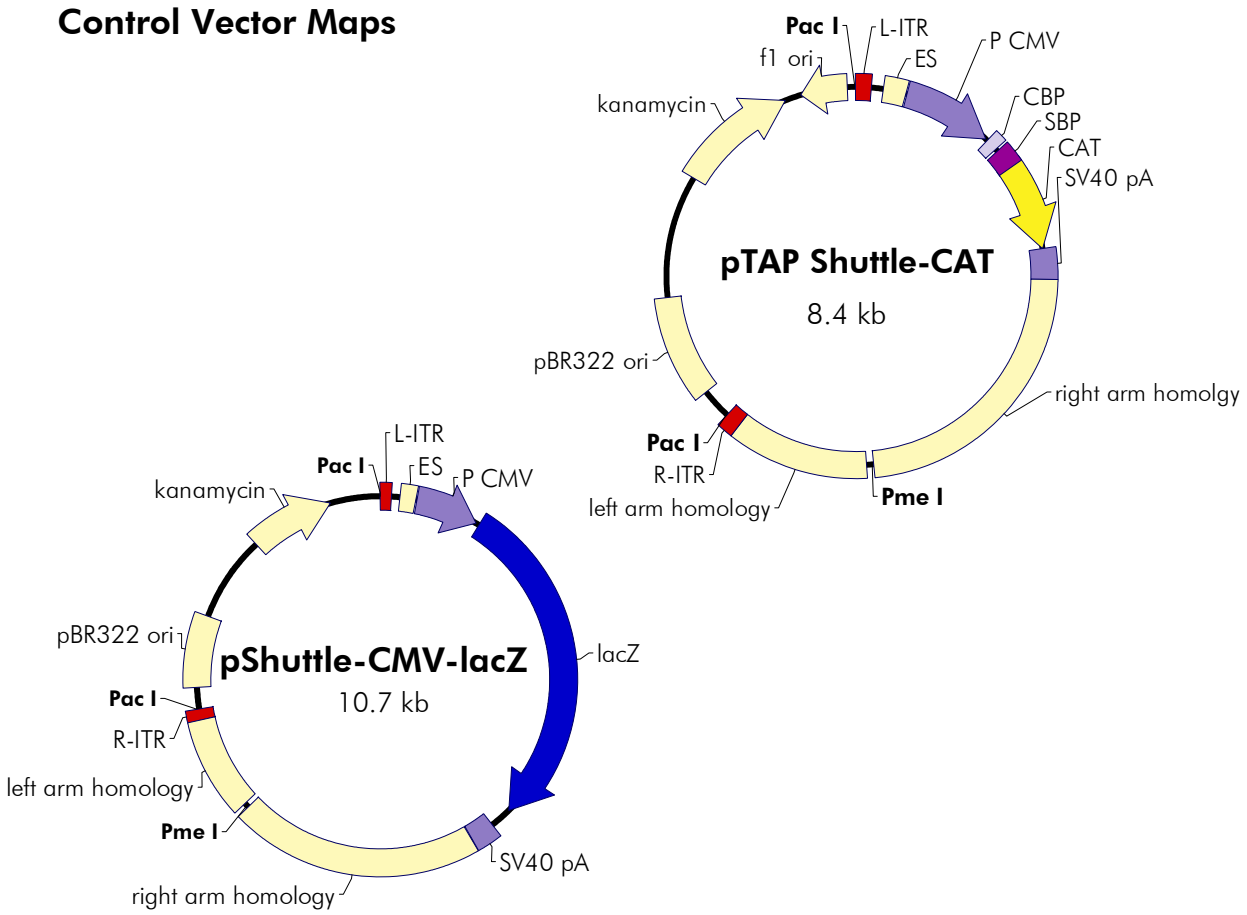


FIGURE 8 Circular maps of the control vectors.

Recommended Primer Sequences

Recommended sequences of primers flanking the MCS suitable for PCR amplification and/or sequencing applications are given in Table II.

TABLE II

Vector	Direction	Sequence
pNTAP Shuttle	**Forward**	5′ GGTCTATATAAGCAGAGCTG 3′
	Reverse	5′ GTGGTATGGCTGATTATGATCAG 3′
pCTAP Shuttle	**Forward**	5′ GGTCTATATAAGCAGAGCTG 3′
	Reverse	5′ GTGGTATGGCTGATTATGATCAG 3′

FEATURES OF BACTERIAL STRAINS

In order to produce and amplify recombinant adenovirus with this adenoviral system, two different prokaryotic host strains are required. The first, BJ5183-AD-1, is the BJ5183 strain pre-transformed with the pAdEasy-1 vector. The BJ5183-AD-1 strain is *recA* proficient and supplies the machinery necessary to execute the recombination event between the shuttle vector and the pAdEasy vector. The efficiency of recovery of recombinant vectors is greatly improved by using the BJ5183-AD-1 strain that is pre-transformed with the pADEasy-1 vector—up to 80–90% of kanamycin resistant colonies are recombinants using this single transformation system, compared to 20% recombinants using the traditional cotransformation procedure.

The second strain, provided as XL10-Gold* ultracompetent cells, is used to amplify the recombined adenovirus vector. This strain is both endonuclease deficient (*endA1*) and recombination deficient (*recA*). The *endA1* mutation greatly improves the quality of plasmid miniprep DNA, and the *recA* mutation helps ensure insert stability.

In the following Table III, the genes indicated in italics signify that the bacterium carries a mutant allele. The genes present on the F′ episome represent the wild-type bacterial alleles.

TABLE III

Host strain	References	Genotype
BJ5183-AD-1 electrocompetent cells	11	*end*A1 *sbc*BC *rec*BC *gal*K *met thi*-1 *bio*T *hsd*R (Str^R) [pAdEasy-1 (Amp^R)]
XL10-Gold ultracompetent cells	12, 13	Tet^R Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* Hte [F′ *proAB lacI*^{q}ZΔ*M15* Tn*10* (Tet^R) Amy Cam^R]

* U.S. Patent Nos.5,512,468, 5,707,841, 6,706,525.

Cloning Considerations

Refer to Figures 6 and 7 for circular maps and corresponding MCS sequences for the adenoviral TAP shuttle vectors and to *www.genomics.agilent.com/vectorMapsAndSequence.jsp* for the complete nucleotide sequence and restriction maps. Each vector sequence has been verified for accuracy at the cloning junctions. The remainder of the sequence of each of the AdEasy vectors has been compiled from existing data.

Absence of *Pme* I and *Pac* I Sites in the Insert DNA

The shuttle vector must be linearized using *Pme* I before transformation of BJ5183-AD-1 bacteria and the recombinant Ad plasmid must be digested with *Pac* I before transfection of the AD-293 packaging cell line. Ensure that the gene of interest does not contain either of these restriction sites. If these sites exist in the gene of interest, it will be necessary to perform site-directed mutagenesis before proceeding to the cloning steps.

Cloning Capacity of the Adenoviral TAP Shuttle Vectors

It is important to adhere to the upper size limit of insert DNA (cloning capacity) of 6.6 kb. Inserting larger fragments results in considerable decreases in efficiency.

Cloning the Gene of Interest

Note *When growing cells harboring either TAP shuttle vector, incubate the cells at 30°C, in order to reduce any unwanted recombinants or potential toxicity. Expect the cells to exhibit slower growth at 30°C, as opposed to 37°C.*

1. Clone the gene of interest into the TAP shuttle vector of choice using appropriate site(s) in the MCS.

2. Confirm the presence of the insert by restriction digestion or sequence analysis.

3. Purify DNA (TAP shuttle vector plus gene of interest) in sufficient quantity for the subsequent *Pme* I-digestion and transformation steps (0.03–0.1 µg of linearized shuttle vector DNA is required for each transformation reaction).

4. Linearize TAP shuttle vector DNA using *Pme* I and confirm complete digestion by agarose gel electrophoresis. It is necessary to prepare linearized samples of the test vector DNA (TAP shuttle vector plus gene of interest), the pShuttle-CMV-*lacZ* control shuttle vector, and the pTAP Shuttle-CAT negative control vector.

5. Remove the enzyme and buffer using a method of choice (for example, using the StrataPrep PCR Purification Kit.)

6. Treat the purified DNA with alkaline phosphatase for 30 minutes at 37°C.

   Note *We have achieved very similar recombination efficiencies using* Pme *I-digested vector that is not dephosphorylated.*

7. Gel-purify the linearized vectors using agarose gel electrophoresis. Include samples of uncut shuttle vector in adjacent lanes for comparison. Ensure that the gel is run long enough to visualize good separation between uncut and cut DNA. Isolate the linearized shuttle vector from the gel using a method of choice. Resuspend the purified DNA in sterile water at a final concentration of ~0.05 µg/µl.

   Notes *Ensure that the method chosen to purify the shuttle vector results in a DNA sample that does not contain salts, which will severely damage cells during electroporation.*

   Direct transformation of BJ5183-AD-1 cells with Pme *I-digested shuttle vector, without dephosphorylation or gel purification, often produces a sufficiently high recombination efficiency to allow recovery of recombinant adenoviral vectors. Longer digestion times (several hours to overnight) produce a greater proportion of linearized vector and increase the recombination efficiency. Transform with 1 µl (~0.05 µg) of digested DNA, after heat-inactivation of the enzyme.*

Transformation Guidelines for BJ5183-AD-1 Cells

Storage Conditions

Electroporation competent cells are sensitive to even small variations in temperature and must be stored at the bottom of a –80°C freezer. Transferring tubes from one freezer to another may result in a loss of efficiency. Electroporation competent cells should be placed at –80°C directly from the dry ice shipping container. When aliquoting, keep electroporation competent cells on ice at all times.

Transforming the BJ5183-AD-1 Cells to Produce Recombinant Ad Plasmid

Note *In this portion of the protocol, the BJ5183-AD-1 cells are transformed with the linearized vectors in individual transformation reactions. A recombination event that takes place in the bacterial cells results in the production of recombinant AdEasy plasmid DNA.*

1. Prechill four DNase-free microcentrifuge tubes and four electroporation cuvettes (0.2 cm gap) on ice.

2. Remove two 100 µl aliquots of BJ5183-AD-1 electroporation competent cells from –80°C storage and thaw on ice.

3. Gently pipet 40 µl of the competent cells into each of the chilled microcentrifuge tubes.

4. Into one of the tubes, pipet 1 µl (~0.05 to 0.1 µg) of linearized TAP shuttle vector. Mix by tapping the tube gently and keep on ice.

5. Into the second tube, pipet 1 µl (~0.05 to 0.1 µg) of linearized pShuttle-CMV-*lacZ* vector. Mix by tapping the tube gently and keep on ice.

6. Into the third tube, pipet 1 µl (~0.05 to 0.1 µg) of linearized pTAP Shuttle-CAT vector. Mix by tapping the tube gently and keep on ice.

7. Into the fourth tube, pipet, 1 µl of Transformation Control plasmid (supplied with the BJ5183-AD-1 electroporation comp. cells).

8. Set the electroporator to the following settings by referring to the instructions provided with the instrument: 200 Ω, 1.7 kV, 25 µF.

9. Transfer the contents of one microcentrifuge tube into one of the chilled electroporation cuvettes and tap the cuvette gently to settle the mixture to the bottom.

10. Slide the cuvette into the electroporation chamber until the cuvette connects with the electrical contacts.

11. Pulse the sample once, then quickly remove the cuvette. **Immediately** add 1 ml of sterile LB broth[§] and pipet up and down to resuspend the cells.

12. Transfer the cell suspension to a sterile 14-ml BD Falcon polypropylene round-bottom tube.

13. Repeat the electroporation for the other three transformation reactions. Incubate all of the transformations at 30°C for 1 hour while shaking at 225–250 rpm.

14. For the recombination reactions (linearized DNA transformants), plate the entire volume of recovered cells on LB-kanamycin agar plates[§] (e.g. three plates containing 50 µl, 100 µl, and 850 µl of the transformed cell suspension, respectively).

    For the transformation using the Transformation Control plasmid, plate 10 µl and 100 µl of the recovered cells on LB-kanamycin agar plates.

    Note *When plating less than 100 µl, first place a 100-µl pool of LB broth on an LB-kanamycin agar plate. Pipet the transformed cells into the pool of LB broth, then use a sterile spreader to spread the mixture.*

15. Incubate the plates for 24–48 hours at 30°C.

[§] See *Preparation of Media and Reagents.*

Testing Colonies for Recombinant Ad Plasmids

1. Examine the transformation plates. The linear vector transformants will appear as three populations: very large colonies, intermediate-, and small-sized colonies. The small and intermediate colonies are the potential recombinants and the very large colonies represent background from the TAP shuttle vector. The ratio of small plus intermediate colonies to very large colonies should be approximately 10:1.

   Note *If the transformation was performed with non-gel purified shuttle vector, expect a ratio of small plus intermediate colonies to very large colonies of 5:1 or lower.*

2. Count colonies obtained on the Transformation Control plasmid transformation plates to determine transformation efficiency, if desired. Expect an efficiency of $\geq 1 \times 10^7$ cfu/µg Transformation Control DNA.

3. Pick 10 or more of the **smallest**, well isolated colonies each from the test recombination plate (TAP shuttle vector plus gene of interest), the adenoviral production control recombination plate (pShuttle-CMV-*lacZ*), and the negative TAP control recombination plate (pTAP Shuttle-CAT) into 3–5 ml cultures of LB-kanamycin broth.[§]

   Note *The recombinants will be low copy number plasmids approximately 40 kb in size. As implied by the small colony size on the test plates, cultures will grow slowly and plasmid yields will be low. Miniprep procedures should be adapted accordingly. Procedures suitable for purification of cosmids or large plasmids are recommended.*

4. Incubate the cultures at 30°C overnight while shaking at 225–250 rpm.

5. Prepare miniprep DNA from 2 ml (or more) of overnight culture using a procedure suitable to purify large plasmids or cosmids. Resuspend the miniprep DNA in 50 µl of sterile dH_2O or TE buffer.[§]

6. Cut 10 µl of the miniprep DNA with *Pac* I restriction enzyme and run the entire digest on a 0.8% agarose TAE[§] gel next to 10 µl of uncut miniprep DNA. As a control, also cut a small amount (~0.2 µg) of unrecombined TAP shuttle vector (prepared in step 3 of *Cloning the Gene of Interest*) and run in an adjacent lane.

[§] See *Preparation of Media and Reagents.*

Interpretation of Results

Restriction of recombinant Ad plasmid DNA with *Pac* I should yield a large fragment of ~30 kb*, and a smaller fragment of either 3.0 kb (if recombination took place between the left arms) or 4.5 kb (if recombination took place at the origins of replication). Uncut recombinants will give a large smear at the top of the gel very close to the wells (and often have a smaller band that runs just below 23 kb). Potential recombinants may be difficult to identify (in some instances you can only visualize the 30 kb band) if the quality or yield of the miniprep is low. If this is the case, prepare DNA from a greater volume of culture with a procedure adapted for purification of large plasmids or cosmids.

Note *Remember to reserve a small amount of each recombinant Ad plasmid DNA miniprep sample for transformation in the subsequent protocol.*

More than 90% of the DNA minipreps should contain a recombinant Ad plasmid close to 30 kb, when small colonies are selected for analysis.

There are often faint background bands in BJ5183-AD-1 minipreps. If the PREDOMINANT bands are the expected sizes, redigest potential recombinant DNA prepared from XL10-Gold cells (see *Amplifying Recombinant Ad Plasmids*). If after that second preparation there are still bands that are unaccounted for, discard the clone. For this reason it is recommended that more than one potential recombinant be amplified in XL10-Gold cells.

* Due to limitations in the resolution of large DNA fragments on 0.8% agarose gels, the 30 kb band can be observed to migrate next to the 23 kb marker of the λ Hind III DNA size ladder.

AMPLIFYING RECOMBINANT AD PLASMIDS

In this part of the protocol, individual positive recombinant Ad plasmids, identified by restriction digest, are used to transform XL10-Gold ultracompetent cells so that the recombinant adenovirus plasmid DNA can be amplified. It is possible that some clones identified as positive by restriction digest will produce low titers of packaged virus or will express the gene of interest poorly. **It is recommended that more than one positive clone be used to transform XL10-Gold cells so that a few individuals can be tested in human cell transfection.**

Transformation Guidelines for XL10-Gold Ultracompetent Cells

Storage Conditions

Ultracompetent cells are sensitive to even small variations in temperature and must be stored at the bottom of a –80°C freezer. Transferring tubes from one freezer to another may result in a loss of efficiency. Ultracompetent cells should be placed at –80°C directly from the dry ice shipping container.

Aliquoting Cells

When aliquoting, keep ultracompetent cells on ice at all times. It is essential that the 14-ml BD Falcon polypropylene tubes are placed on ice before the cells are thawed and that the cells are aliquoted directly into the prechilled tubes. It is also important to use at 100 µl of ultracompetent cells/transformation. Using a smaller volume will result in lower efficiencies.

Use of 14-ml BD Falcon Polypropylene Tubes

It is important that 14-ml BD Falcon polypropylene round-bottom tubes are used for the transformation protocol, since other tubes may be degraded by the β-mercaptoethanol used in step 3 of the *Transformation Protocol*. In addition, the incubation period during the heat-pulse step is critical and has been optimized specifically for the thickness and shape of 14-ml BD Falcon polypropylene tubes.

Use of β-Mercaptoethanol

β-Mercaptoethanol (β-ME) has been shown to increase transformation efficiency. The XL10-Gold β-mercaptoethanol mix provided in this kit is diluted and ready to use. For optimum efficiency, use 4 µl of the β-ME mix. (Using an alternative source of β-ME may reduce transformation efficiency.)

Length and Temperature of the Heat Pulse

There is a defined window of highest efficiency resulting from the heat pulse during transformation. Optimal efficiencies are observed when cells are heat-pulsed for 30 seconds. Do not exceed 42°C.

XL10-Gold Ultracompetent Cells Transformation Protocol

Note *Each 100 µl aliquot of XL10-Gold ultracompetent cells is sufficient for one transformation. Thaw as many aliquots as required to transform the test recombinant adenovirus plasmid(s) (shuttle vector plus gene of interest recombined with pAdEasy-1) and the control recombinant adenovirus plasmids (pShuttle-CMV-*lacZ *and pTAP Shuttle-CAT, each recombined with pAdEasy-1). It is recommended that more than one test recombinant adenovirus plasmid be transformed in this step.*

When growing cells harboring either TAP shuttle vector, incubate the cells at 30°C, in order to reduce any unwanted recombinants or potential toxicity. Expect the cells to exhibit slower growth at 30°C, as opposed to 37°C.

1. Prepare a 42°C water bath.
2. Prechill 14-ml BD Falcon polypropylene tubes on ice.
3. Thaw the XL10-Gold ultracompetent cells on ice.
4. Gently mix the cells by hand. Aliquot 100 µl of the cells into prechilled 14-ml tubes.
5. Add 4 µl of the β-ME mix provided with the kit to the 100 µl of cells. (Using an alternative source of β-ME may reduce transformation efficiency.)
6. Swirl the contents of the tube gently. Incubate the cells on ice for 10 minutes, swirling gently every 2 minutes.
7. For each plasmid to be transformed, add 0.1–50 ng of DNA (generally 1 µl of miniprep DNA, with a maximum volume of 4 µl) to an aliquot of cells and swirl gently. To a separate tube of cells add 1 µl of the pUC18 control plasmid (diluted 1:10 in sterile dH_2O) and swirl gently.
8. Incubate the tubes on ice for 30 minutes.
9. Prewarm NZY$^+$ broth (see *Preparation of Media and Reagents*) in a 42°C water bath for use in step 12.

   Note *Transformation of XL10-Gold ultracompetent cells has been optimized with* ***NZY$^+$ broth.***

10. Heat-pulse the tubes in a 42°C water bath for 30 seconds. The duration of the heat pulse is *critical* for obtaining the highest efficiencies. Do not exceed 42°C.
11. Incubate the tubes on ice for 2 minutes.

12. Add 0.9 ml of prewarmed (42°C) NZY$^+$ broth to each tube and incubate the tubes at 30°C for 1 hour with shaking at 225–250 rpm.

13. Plate 5 µl, 25 µl, and 100 µl of each putative recombinant transformation reaction on LB-kanamycin agar plates using a sterile spreader.

    Plate 5 µl of the pUC18 control plasmid transformation on LB-ampicillin agar plates (see *Preparation of Media and Reagents*). The pUC18 control transformation should yield an efficiency of > 10^9 cfu/µg pUC 18 DNA.

    Note *When plating less than 100 µl, first place a 100-µl pool of NZY$^+$ broth on the agar plate. Pipet the transformed cells into the pool of NZY$^+$ broth, then use a sterile spreader to spread the mixture.*

14. Incubate the plates for 48 hours at 30°C.

15. Pick one colony from each transformation and transfer into 10 ml of LB-kanamycin broth. Grow the cultures overnight at 30°C while shaking at 225–250 rpm.

16. The next morning transfer 5 ml of each overnight culture into a clean tube and store at 4°C.

17. **(OPTIONAL)** Prepare miniprep DNA from some or all of the remaining 5 ml using any standard miniprep procedure. Digest 5 µl of the miniprep DNA with *Pac* I and analyze by agarose gel electrophoresis (using a 0.8% agarose TAE gel). Confirm the desired restriction pattern (one band of ~30 kb and a second band of either 3.0 kb or 4.5 kb) prior to inoculating a 500-ml flask in step 18.

18. Use the 5 ml of overnight culture stored at 4°C to inoculate a flask containing 500 ml LB-kanamycin broth. Grow the culture overnight at 30°C while shaking at 225–250 rpm.

19. The following morning, prepare maxiprep DNA from the liquid culture. This DNA will be used to transfect human cells and must be of suitable quality and purity (e.g. prepared using standard cesium chloride density gradient centrifugation or affinity column purification that produces DNA of equivalent quality).

20. Digest a sufficient amount of each purified recombinant adenovirus plasmid with *Pac* I. (5 µg of DNA is needed for each transfection.)

21. Run 0.2 µg of each cut DNA on a 0.8% agarose TAE gel and confirm the desired restriction pattern (one band ~30 kb and a second band of either 3.0 kb or 4.5 kb).

22. Remove buffer and enzyme from the remainder of the restriction reactions by phenol extraction/ethanol precipitation or using a similar DNA purification kit (e.g. the StrataPrep PCR Purification Kit).

23. Under sterile conditions, resuspend the DNA in 50 µl of sterile 0.1× TE buffer (see *Preparation of Media and Reagents*) or dH_2O. Store the resuspended DNA at –20°C.

AD-293 CELL CULTURE GUIDELINES

Notes *All procedures must be performed using sterile technique in a laminar flow hood. For general information on mammalian cell culture and sterile technique, see reference [15].*

AD-293 cells may be passaged up to 30 times (with the supplied cells defined as passage number one). It is important to prepare a liquid nitrogen stock of early passage cell aliquots for long-range experiments.

Despite the improved adherence of AD-293 cells, it is important to minimize monolayer disruption during passaging and plaque assays by gently pipetting liquids down the side of the culture dish instead of pipetting directly onto the cells.

Establishing AD-293 Cultures from Frozen Cells

1. Place 10 ml of growth medium (see *Preparation of Media and Reagents*) in a 15-ml conical tube.

2. Thaw the frozen cryovial of cells within 40–60 seconds by gentle agitation in a 37°C water bath. Remove the cryovial from the water bath and decontaminate the cryovial by immersing it in 70% (v/v) ethanol (at room temperature).

3. Transfer the thawed cell suspension to the conical tube containing 10 ml of growth medium.

4. Collect the cells by centrifugation at 200 × *g* for 5 minutes at room temperature. Remove the growth medium by aspiration.

5. Resuspend the cells in the conical tube in 5 ml of fresh growth medium.

6. Add 10 ml of growth medium to a 75-cm^2 tissue culture flask. Transfer the 5 ml of cell suspension to the same tissue culture flask. Place the cells in a 37°C incubator at 5% CO_2.

7. Monitor cell density daily. Cells should be passaged when the cell culture is at ≤50% confluency. Proceed to either *Preparation of an AD-293 Cell Liquid Nitrogen Stock* or *Passaging of AD-293 Cells.*

Preparation of an AD-293 Cell Liquid Nitrogen Stock

1. When growing cells for the production of an AD-293 liquid nitrogen stock, cultures should be maintained at ≤50% confluence.

   Note *AD-293 cells grown at high confluence may lose the increased adherence phenotype. It is especially important to maintain cells propagated to establish a liquid nitrogen stock at≤50% confluence to ensure the integrity of the stock.*

2. Collect cells from a healthy, log-phase culture. Remove the culture medium by aspiration. Trypsinize cells for 1–3 minutes in 1.5 ml of Trypsin-EDTA Solution.[§]

   Note *Incubate the cells in the Trypsin-EDTA solution for the minimum time required to release adherent cells from the flask. This process may be monitored using an inverted microscope. Excess trypsinization may damage or kill the cells.*

3. Dilute the cells with 8.5 ml of growth medium. The serum in the medium inactivates the trypsin. Transfer the suspension to a 15-ml conical tube, then collect the cells by centrifugation at 600 × *g* for 5 minutes at room temperature.

4. Remove the medium by aspiration. Resuspend the cell pellet in a minimal volume of growth medium (containing 10% fetal bovine serum). Count the cells present in an aliquot of the resuspension using a hemocytometer.

5. Dilute the cell suspension to 1×10^6 cells/ml in freezing medium,[§] then dispense 1-ml aliquots of the suspension into 2-ml cryovials.

6. Freeze the cell aliquots gradually by placing the vials in a Styrofoam® container and then placing the container in a –80° freezer overnight.

7. Transfer the vials of frozen cells to liquid nitrogen for long-term storage.

Passaging of AD-293 Cells

When the cell monolayer reaches 50% confluence, AD-293 cells should be split at a 1:10 ratio.

Note *If cell confluence exceeds 50%, AD-293 cells may lose the increased adherence phenotype.*

1. Remove the growth medium by aspiration. Wash cells once with 10 ml of phosphate-buffered saline.

2. Trypsinize cells for 1–3 minutes in 1.5-ml of Trypsin-EDTA Solution.

3. Dilute the cells with 8.5 ml of growth medium to inactivate the trypsin.

4. Transfer 1 ml of the cell suspension to a fresh 75-cm^2 tissue culture flask and add 9 ml fresh growth medium. Place the cells in a 37°C incubator at 5% CO_2. Monitor cell density daily.

[§] See *Preparation of Media and Reagents.*

PREPARATION OF PRIMARY ADENOVIRUS STOCK WITH RECOMBINANT AD PLASMID

Safety Considerations

Note *The safety guidelines presented in this section are not intended to replace the BSL 2+ safety procedures already in place at your facility. The information set forth below is intended as an additional resource and to supplement existing protocols in your laboratory.*

Prior to use of the AdEasy vectors, we strongly recommend that users become thoroughly familiar with the safety considerations concerning the production and handling of adenovirus. For a description of laboratory biosafety level criteria, consult the Centers for Disease Control Office of Health and Safety Web site *http://www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4s3.htm*. Production of adenovirus and use of adenoviral vectors fall within NIH Biosafety Level 2 criteria. For more information regarding BSL-2+ practices, consult the UCSD Environmental Health and Safety Web site *http://www-ehs.ucsd.edu/ADENO.HTM*.

Note *The steps performed in this section,* Preparation of Primary Adenovirus Stock with Recombinant Ad Plasmid, *need to be carried out under sterile conditions in a laminar flow hood which is designated for use with virus. For handling adenovirus-containing solutions, using disposable pipets or pipettors with filter tips to prevent the transfer of contaminated aerosols is strongly recommended.*

Description of AD-293 Cells

Prepare and titer AdEasy recombinant virus stocks using the AD-293 cell line [provided with the InterPlay adenoviral TAP system and available separately (Catalog #240085)]. AD-293 cells are derived from the commonly used HEK293 cell line, but have improved cell adherence and plaque formation properties. HEK293 cells are human embryonic kidney cells transformed by sheared adenovirus type 5 DNA.[16] AD-293 cells, like HEK293 cells, produce the adenovirus E1 gene in *trans,* allowing the production of infectious virus particles when cells are transfected with E1-deleted adenovirus vectors such as the pAdEasy-1 vector. Standard HEK293 cells do not adhere well to tissue culture dishes, hindering adherent cell culture and plaque assay procedures. AD-293 cells demonstrate improved adherence to tissue culture dishes, making AD-293 cell monolayers less susceptible to disruption.

Note *Despite the improved adherence of AD-293 cells, it is important to minimize monolayer disruption during passaging and plaque assays by gently pipeting liquids down the side of the culture dish instead of pipetting directly onto the cells.*

Preparing AD-293 Cells for Transfection

Plate AD-293 cells at 7–8 × 10^5 cells per 60-mm tissue culture dish in growth medium 24 hours prior to the transfection.

Note *To achieve optimal titers, it is important that the AD-293 cells are healthy and plated at optimal density. Cells should be passaged at ≤50% confluence, and ideally passaged no more than 30 times. It is thus prudent to initially prepare a large number of vials of the cells while they are at a low passage and healthy, storing the vials in liquid nitrogen for long-range experiments. Care should be taken to avoid clumping of the cells during passaging and plating for transfection.*

Transfecting AD-293 Cells

Perform this procedure immediately prior to transfection.

Notes *A variety of transfection protocols may be successfully used with these vectors and the AD-293 cell line. We have demonstrated success using the following modification of the Agilent GeneJammer Transfection Reagent protocol. As a general guideline, the following protocol is expected to produce a viral titer of approximately 10^6–10^7 plaque forming units (pfu)/ml when transducing AD-293 cells.*

A visible precipitate may be seen in the GeneJammer transfection reagent solution. Incubate the vials at room temperature to resolubilize the precipitate before use.

1. Transfer 250 µl of sterile, room temperature, serum-free, antibiotic-free DMEM (or medium of choice) to a polystyrene tube.

2. Add 15 µl of the GeneJammer transfection reagent (3 µl/µg DNA) by pipetting directly into serum-free medium. **The presence of serum at this stage is inhibitory**. To mix, stir gently with the pipet tip. Do not vortex.

   Note *Avoid allowing the undiluted reagent to contact the plastic tube directly. This greatly reduces the efficacy of the reagent.*

3. Incubate at room temperature for 5–10 minutes.

4. For each transfection, pipet 5 µg of the *Pac I* digested, recombinant Ad plasmid DNA into the diluted GeneJammer transfection reagent and mix gently.

5. Incubate for 5–10 minutes at room temperature.

6. Remove the standard culture medium from the tissue culture dish by aspiration and replace it with 2.25 ml of fresh serum-containing medium.

7. Add the transfection mixture dropwise to the tissue culture dish. Gently rock the dish back and forth to distribute the transfection mixture evenly. Incubate the dish in standard growth conditions (i.e., 37°C and 5% CO_2 in a humidified incubator).

8. After 3 hours add an equal volume of serum-containing medium.

   Note *From this point on, it should be assumed that adenovirus is present in plates containing the transfected cells. Gloves and disposable lab coats should be worn while working with the virus. When pipetting solutions and transferring plates to and from the laminar flow hood, contamination with aerosols should be avoided. In case of spills, follow the procedures recommended at your facility. Additionally, consult the Web sites described in* Safety Considerations.

9. Incubate the culture plates at 37°C for 7–10 days, replenishing the growth medium when needed (based on media color). If the cells appear to be well attached to the plate, replace the medium with 4 ml of fresh medium, taking care not to dislodge the cells. Alternatively, if detached cells are observed in the growth medium, add an equal volume of fresh medium to the existing medium. These cells will be used to prepare the primary viral stocks.

Preparing the Primary Viral Stocks

1. Prepare a small dry ice-methanol bath and a small 37°C water bath and place them in the laminar flow hood.

2. Carefully remove growth medium from adenovirus-producing AD-293 plates and wash the cells once with PBS (see *Preparation of Media and Reagents*). Take care not to lose any clusters of floating and partially attached cells during this process.

   Note *If the cells are already mostly detached, pipet up and down gently in the growth medium until cells become completely resuspended. Transfer cell suspension to a screw cap centrifuge tube and pellet the cells by low speed centrifugation. Aspirate medium, and wash the cells once with 0.5 ml of sterile PBS. Resuspend the cell pellet in a fresh 0.5 ml sterile PBS (per 60-mM dish) and proceed to Step 5.*

3. Add 0.5 ml of PBS to each plate of cells to be harvested. Collect the cells by holding the plate at an angle and scraping the cells into the pool of PBS with a cell lifter.

4. Transfer the cell suspension to a 1.7-ml screw-cap microcentrifuge tube. If duplicate DNA samples were transfected, the cells from duplicate samples may be combined in the microcentrifuge tube at this stage.

5. Subject the cell suspension to four rounds of freeze/thaw by alternating the tubes between the dry ice-methanol bath and the 37°C water bath, vortexing briefly after each thaw.

6. Collect cellular debris by microcentrifugation at 12,000 × *g* for 10 minutes at room temperature.

7. Transfer the supernatant, which contains the primary virus stock, to a fresh screw-cap microcentrifuge tube. Aliquot and store at –80°C. Viral stocks can be stored for more than one year at –80°C.

GUIDELINES FOR INFECTION CONDITIONS AND AMPLIFICATION OF THE PRIMARY VIRAL STOCK

Titer Considerations

Primary viral stocks produced with the above protocol are generally expected to be in the 10^7–10^8 pfu/ml range. However, there is significant variation in titer achieved based on differences in constructs and recombinant clones, as well as user methodology and fluctuations in transfection efficiency. The titer of the virus stock can be determined by plaque assay (see *Appendix*).

In practice, it generally saves time to proceed with the virus stock amplification, monitor the infection visually (see *Monitoring the Infection*), and then titer the amplified virus stock for use in subsequent applications.

Amplification Guidelines

Amplification of a virus stock is achieved by infection of AD-293 cultures with a low passage virus stock. One round of amplification generally produces a 10-fold increase in titer.

Minimizing the Production of Replication-Competent Adenovirus (RCA)

Since AD-293 cells possess integrated human Ad5 DNA, there is a low frequency of homologous recombination between the E1-deleted vector and the host DNA resulting in the production of some replication competent adenovirus (RCA). The frequency of occurrence is very low, but the percentage of RCA in a given virus stock goes up with each amplification of that stock. **The primary viral stock contains the lowest numbers of RCA, and it is recommended that all amplifications be initiated with virus stock at the lowest possible passage number**.

Infection Procedure Guidelines

Infection of AD-293 cells may be achieved simply by adding a solution of viral particles to adherent cells in tissue culture dishes. To amplify a virus stock, prepare cultures of AD-293 cells that are 50–70% confluent (see *Optimizing Infection Conditions*). Dilute the primary virus stock into a minimal volume of growth medium (just enough to cover cells) and add the virus suspension to the cell culture dishes. Incubate the infection reactions for 2 hours, preferably on a rocking platform to disperse the solution evenly. After two hours, supply additional growth medium to the culture. After the desired number of days of incubation,* harvest the cells in a minimal volume of PBS. Prepare the amplified virus stock by 4 rounds of freeze/thaw as described in *Preparing the Primary Viral Stock*.

* The number of days infection/amplification is allowed to proceed will depend on the confluency of the cells at the time of infection and on the initial ratio of virus particles to cells (multiplicity of infection). See *Monitoring the Infection* for additional information.

Optimizing Infection Conditions

The multiplicity of infection (MOI) is the number of virus per cell used to infect a culture. At high MOIs (10–20), cells should be plated at high densities (near confluence) as once the virus takes over the cell machinery, the cell will cease to divide. The opposite is optimal for low MOI infections; cells should be infected near 50% confluence as only a fraction of the cells will become infected initially and the uninfected cells can continue to grow until they become infected.

Confluence (%)	Cells needed for a 60-mm plate	Cells needed for a 100-mm plate
50%	1.5×10^6	3.5×10^6
75%	2.5×10^6	5.5×10^6
100%	3.5×10^6	7.5×10^6

Monitoring the Infection

To monitor the progress of an adenoviral infection, it is necessary to observe phenotypic changes to infected cells. The cells will show evidence of a cytopathic effect (CPE): cells will round up and detach from the plate, and the nucleus will occupy a major part of the cell due to the high level of virus production. High MOI infections will show complete CPE and can be harvested as soon as three days post-infection, whereas low MOI infections will need to incubate for longer periods until CPE is observed (up to 10 days). The titer of the virus stock can be determined by plaque assay (see *Appendix*).

EXPRESSING THE PROTEIN OF INTEREST IN A CHOSEN CELL LINE

The efficiency of protein expression will vary depending on the cell line and gene of interest. Varying the MOI may be required to achieve optimal gene expression. In most cases, expression of the genes should be detectable 24–72 hours after transduction. A time course is recommended to determine the optimal time to detect gene expression.

Perform the appropriate assays to determine protein expression levels (e.g., Western blotting). An antibody to the CBP peptide is commercially available (Upstate Catalog #07-482).

LACZ CONTROL: DETECTION AND APPLICATIONS

The control vector pShuttle-CMV-*lacZ* can be used to monitor your success at various points during the AdEasy procedure. To detect the presence of LacZ the cells are stained with X-gal. Any in situ X-gal staining procedure will work, but we recommend the In Situ β-galactosidase staining kit (Agilent Catalog #200384). The β-galactosidase activity is easily detected, making this a useful control for the recombinant Ad plasmid transfection.

Transfection Control

A minimum of two days post-transfection with *Pac* I digested LacZ recombinant Ad plasmid, cells can be stained with X-gal to evaluate the success of the transfection. Keep in mind that if transfected into an E1-complementing cell line such as AD-293, adenovirus will be present and the staining procedure should be performed under BSL-2+ guidelines.

Virus Control

An X-gal stain can be performed on adenovirus infected cells to (1) confirm that the transfection was successful and that infectious virus particles were produced (2) estimate titer of stocks produced from transfection or amplification and (3) test the ability of adenovirus vectors to infect a potential target cell. Three days of incubation post-infection is adequate to detect the presence of LacZ.

Lysis buffer

Prepare the lysis buffer by adding protease inhibitors (see guidelines below), then keep the prepared buffer at 4°C. Prepare only the amount of lysis buffer required for one day of experimental work.

For example, for each milliliter of lysis buffer, add 10 µl of a protease inhibitor cocktail (Sigma Catalog #P8340) and 10 µl of 100 mM of PMSF (Sigma Catalog #P7626). The PMSF must be added to the buffer immediately before use. Mix the buffer gently by inverting the tube.

Streptavidin binding buffer (SBB)

Prepare the SBB by adding 7 µl of 14.4 M β-mercaptoethanol (provided) to 10 ml of SBB. Mix the buffer gently by inverting the tube. Best results are obtained when the buffer is used within 24 hours of preparation. However, the prepared buffer may be stored at 4°C for up to two weeks.

Note *To further prevent protein degradation, 100 µl of protease inhibitor cocktail and 100 µl of 100 mM of PMSF per 10 ml of prepared SBB may be added. The PMSF must be added to the buffer immediately before use. Mix the buffer gently by inverting the tube.*

Streptavidin elution buffer (SEB)

The SEB must be prepared and kept at 4°C and protected from light. Prepare only the amount of buffer required for one day of experimental work. Prepare the SEB by adding 7 µl of 14.4 M β-mercaptoethanol (provided) to 10 ml of SEB. Mix the buffer gently by inverting the tube. Best results are obtained when the buffer is used within 24 hours of preparation.

Note *To further prevent protein degradation, 100 µl of protease inhibitor cocktail and 100 µl of 100 mM of PMSF per 10 ml of prepared SEB may be added. The PMSF must be added to the buffer immediately before use. Mix the buffer gently by inverting the tube.*

Calmodulin binding buffer (CBB)

Prepare the CBB by adding 7 µl of 14.4 M β-mercaptoethanol (provided) to 10 ml of CBB. Mix the buffer gently by inverting the tube. Best results are obtained when the buffer is used within 24 hours of preparation. However, the prepared buffer may be stored at 4°C for up to two weeks.

Note *To further prevent protein degradation, 100 µl of protease inhibitor cocktail and 100 µl of 100 mM of PMSF per 10 ml of prepared CBB may be added. The PMSF must be added to the buffer immediately before use. Mix the buffer gently by inverting the tube.*

Calmodulin elution buffer (CEB)

Prepare the CEB by adding 7 µl of 14.4 M β-mercaptoethanol (provided) to 10 ml of CEB. Mix buffer gently by inverting the tube. Best results are obtained when the buffer is used within 24 hours of preparation. However, the prepared buffer may be stored at 4°C for up to two weeks.

Note *Do not add protease inhibitor to the CEB. The protease inhibitor proteins will interfere with subsequent analysis of the TAP-purified proteins.*

TAP PROTOCOL

Notes *Perform all protein purification steps at 4°C to prevent the interacting proteins from dissociating.*

The quantities of the reagents given are recommended for processing 1 × 10^7 cells or 1 × 10^8 cells.

It is recommended to use non-stick tubes during the purification procedure to reduce sample loss due to absorption to the tube walls. This is especially relevant during the calmodulin-resin binding and elution steps when protein concentrations can be relatively low.

When performing the TAP purification protocol using HeLa cells, for example, it is recommended to process cells from at least 10 T-175 flasks (containing approximately 1 x 10^7 cells each) in order to obtain sufficient amounts of protein for mass spectrometry analysis. A single T-175 flask yields sufficient protein for Western blot analysis.

When processing multiple flasks, cell preparations may be scaled-up, pooled, and processed concurrently.

Preparing the Protein Extracts

Step #	Protocol step	For processing 1 × 10^7 cells	For processing 1 × 10^8 cells
1	Resuspend the cells in lysis buffer in a polypropylene centrifuge tube. Subject the cells to three successive rounds of freeze-thawing by incubating the cells on dry ice for 10 minutes or in a –80°C freezer for 20 minutes to freeze the cells, followed by incubating the cells for 10 minutes in cold water to thaw the cells.	1 ml lysis buffer	10 ml lysis buffer
2	Centrifuge the cells at 16,000 × g for 10 minutes to pellet the cell debris. Collect the supernatant.	—	—
3	Transfer a small aliquot of the supernatant to a microcentrifuge tube. Store the aliquot at –20°C for later Western blot analysis (load 5 µl per lane).	—	—
4	To each milliliter of cell lysate, add 0.5 M EDTA and 14.4 M β-mercaptoethanol (provided).	4 µl 0.5 M EDTA 0.7 µl 14.4 M β-ME	40 µl 0.5 M EDTA 7 µl 14.4 M β-ME

Preparing the Streptavidin Resin

Step #	Protocol step	For processing 1×10^7 cells	For processing 1×10^8 cells
5	Centrifuge the 50% streptavidin resin slurry at 1500 × g for 5 minutes to collect the resin. Discard the supernatant to remove the ethanol storage buffer. Resuspend the resin in 1 ml of SBB. When processing multiple preps, the resin can be pooled and washed in 1 ml of SBB. Repeat this wash step.	50 µl 50% streptavidin resin slurry (25 µl resin) 1 ml SBB/wash	500 µl 50% streptavidin resin slurry (250 µl resin) 1 ml SBB/wash
6	Collect the resin by centrifugation at 1500 × g for 5 minutes. Discard the supernatant and resuspend the resin in SBB.	25 µl SBB	250 µl SBB

Purifying the Protein Complexes Using Streptavidin Resin

Step #	Protocol step	For processing 1×10^7 cells	For processing 1×10^8 cells
7	Add the washed streptavidin resin (50% slurry) to the lysate from step 4.	50 µl washed resin (50% slurry)	500 µl washed resin (50% slurry)
8	Rotate the tube at 4°C for 2 hours to allow the tagged proteins to bind to the resin.	—	—
9	Collect the resin by centrifugation at 1500 × g for 5 minutes. Remove and freeze the supernatant for possible analysis later. Resuspend the resin in 1 ml of SBB by rotating the tube at 4°C for 5 minutes. Repeat this wash step.	1 ml SBB/wash	1 ml SBB/wash
10	Collect the resin by centrifugation at 1500 × g for 5 minutes. Discard the supernatant. Add SEB to the resin.	100 µl SEB	1 ml SEB
11	Rotate the tube at 4°C for 30 minutes to elute the protein complexes.	—	—
12	Collect the resin by centrifugation at 1500 × g for 5 minutes. Carefully transfer the supernatant to a fresh tube (the supernatant contains the eluted proteins).	—	—
13	Transfer an aliquot of the cell supernatant to a microcentrifuge tube. Store the aliquot at –20°C for later Western Blot analysis (load 10 µl per lane).	—	—
14	Add the streptavidin supernatant supplement to the supernatant. Then, add CBB to the supplemented supernatant.	2 µl streptavidin supernatant supplement 400 µl CBB	20 µl streptavidin supernatant supplement 4 ml CBB

Preparing the Calmodulin Resin

Step #	Protocol step	For processing 1×10^7 cells	For processing 1×10^8 cells
15	Centrifuge the 50% calmodulin resin slurry at 1500 × g for 5 minutes to collect the resin. Discard the supernatant to remove the ethanol storage buffer. Resuspend the resin in 1 ml of CBB. When processing multiple preps, the resin can be pooled and washed in 1 ml CBB. Repeat this wash step.	25 µl 50% calmodulin resin slurry (12.5 µl resin) 1 ml CBB/wash	250 µl 50% calmodulin resin slurry (125 µl resin) 1 ml CBB/wash
16	Collect the resin by centrifugation at 1500 × g for 5 minutes. Discard the supernatant and resuspend the resin in CBB.	12.5 µl CBB	125 µl CBB

Purifying the Protein Complexes Using Calmodulin Resin

Step #	Protocol step	For processing 1×10^7 cells	For processing 1×10^8 cells
17	Add the washed calmodulin resin (50% slurry) to the eluted proteins from step 14.	25 µl washed resin (50% slurry)	250 µl washed resin (50% slurry)
18	Rotate the tube at 4°C for 2 hours to allow the protein complexes to bind to the resin.	—	—
19	Centrifuge the resin at 1500 × g for 5 minutes. Remove and freeze the supernatant for possible analysis later. Resuspend the resin in 1 ml of CBB by rotating the tube at 4°C for 5 minutes. Repeat this wash step.	1 ml CBB/wash	1 ml CBB/wash
20	Collect the resin by centrifugation at 1500 × g for 5 minutes. Discard the supernatant. Proceed to steps 21a or 21b to separate the protein complexes from the calmodulin resin, depending on the downstream methods of analysis chosen. If the purified protein is required to be in native configuration for subsequent applications, such as determination of enzymatic activity, elute the purified protein from the calmodulin resin as described in step 21a. If the protein is analyzed using other methods, such as mass spectroscopy or immunoblotting, proceed to the boiling method in step 21b.	—	—

Step #	Protocol step	For processing 1×10^7 cells	For processing 1×10^8 cells
21a	**Elution method:** To separate the protein complexes from the resin by elution, add CEB to the collected calmodulin resin. Rotate the tube at 4°C for 30 minutes to elute the protein complexes. Collect the resin by centrifugation at 1500 × g for 5 minutes. Carefully transfer the supernatant to a fresh tube. The supernatant contains the Tandem Affinity Purified protein complexes. Addition of 10% glycerol may help to prevent loss of enzyme activity due to freeze–thaw cycles. The supernatant may be stored at –20°C for future processing.	50 µl CEB	500 µl CEB
21b	**Boiling method:** Resuspend the resin in an equal volume of 1× loading buffer suitable for the separation system used in the subsequent analysis. Boil the resuspended resin for 5 minutes. Carefully transfer the supernatant to a fresh tube. The supernatant contains the Tandem Affinity Purified protein complexes. The supernatant may be stored at –20°C for future processing. It may be necessary to reduce the volume of the supernatant in order to load a sufficient amount on a gel, for analysis by mass spectroscopy. A protocol for concentrating the supernatant is given in the *Appendix*. **Note** *It is essential to use the MS-grade calmodulin resin provided with this procedure.*	—	—

DETECTION OF PURIFIED PROTEINS

TAP-purified proteins may be analyzed by Western blotting and mass spectrometry.

Western Blotting

To detect the purified proteins, resolve the protein preparation by SDS-PAGE. Experimental proteins may be detected using protein-specific antibodies.

Notes *Some TAP-tagged baits may not efficiently elute from the calmodulin resin. In this case, boil the beads for 5 minutes in 1× loading buffer before loading onto the gel.*

Mass Spectrometry

To characterize the TAP-purified proteins, concentrate samples by TCA precipitation and resolve the interacting proteins by SDS-PAGE. Perform in-gel digestion on individual protein bands with trypsin and characterize corresponding peptide finger prints by mass spectrometry.

Using the pTAP Shuttle-CAT Control

The recombinant pTAP Shuttle-CAT adenovirus can be used to generate a set of background proteins for mass spectrometry analysis produced by the TAP purification procedure. To generate this data, transduce the cell line chosen for the protein of interest to be expressed. At least 1 x 10^8 cells are recommended for this process.

Load the purified proteins of interest and the purified CAT proteins on a SDS-PAGE side by side. Perform an in-gel digestion on individual protein bands with trypsin and characterize corresponding peptide finger prints by mass spectrometry.

APPENDIX

Plaque Assay using Agarose Overlay

The following protocol may be used to determine the titer (pfu/ml) of a viral stock. In addition, although the AdEasy system essentially obviates the need to plaque purify clones for a viral stock, if desired, the following protocol can also be used to isolate a single virus clone.

Preparing Viral Stock Dilutions

1. Plate AD-293 cells at a density of 5×10^5 per well of 6-well tissue culture plates.

2. Incubate overnight at 37°C.

3. Dilute viral stocks in 1-ml volumes over a 10-fold series from 10^{-5} to 10^{-9} in growth medium. Carry dilutions in duplicate.

4. Add 1 ml of each dilution to a separate well of the 6-well plate. Leave one well "medium only" (no virus added) as a control.

5. Incubate at 37°C for 2 hours. Gentle rocking during the incubation is beneficial but not required.

6. Proceed to *Overlaying the Infected Cells with Agarose.*

Overlaying the Infected Cells with Agarose

Note *The agarose overlay should be applied so that it spreads fast enough to cover the plate before solidifying but not so fast that the cells are disrupted. It may be necessary to practice the overlaying technique on uninfected cells prior to performing the plaque assay. Prior to the addition of the agarose overlay, inspect the plates containing the cells to ensure adequate adherence.*

1. Prepare a solution of 5% SeaPlaque® agarose* in sterile PBS, autoclave, and store in 10-ml aliquots at 4°C in 50-ml sterile conical tubes.

2. Prior to use, melt the agarose by placing the tube in a beaker of boiling water. Do not microwave the tube of agarose.

3. Once melted, cool the agarose to 45°C.

4. Add 30 ml of growth medium previously equilibrated to 37°C and mix. This makes the final agarose concentration 1.25%. Proceed immediately to the next step.

* SeaPlaque GTG agarose (Catalog #50111) from BioWhittaker Molecular Applications (1-800-341-1574 or *www.bmaproducts.com*).

5. Completely remove the growth medium from the wells that will receive the overlay.

6. Gently pipet 3 ml of agarose/growth medium mix very gently along the side of the well and allow it to completely cover the bottom of the well.

7. Incubate the plate at 37°C. Plaques, having the appearance of small white spots, should be visible to the naked eye within 12–21 days. During that time, if the agarose/growth medium overlay becomes yellow, pour additional overlays at a volume of 1.5 ml per addition.

8. To determine titer, count plaques from wells where isolated plaques are clearly visible and countable. Average the counts from duplicate wells and multiply that number by the dilution factor to estimate pfu/ml.

Plaque Isolation

1. Under sterile conditions, core-out well isolated plaques (as agarose plugs) with a sterile Pasteur pipette and transfer to 250 µl of growth medium in a sterile microcentrifuge tube.

2. Elute virus for 24 hours at 37°C.

3. Use this virus stock to perform an additional round of plaque purification if desired, or amplify for later use.

Concentrating the TAP Protein Complex-containing Supernatant

Sample concentration using Millipore Amicon® Microcon® devices (specifically, the Microcon® YM-10 centrifugal filter unit) is recommended to achieve maximum retention of purified proteins. Consult the manufacturer's product specifications and guidelines prior to use.

Unit Pre-treatment

In order to increase recovery, pre-treat the plastic device before use in order to block available binding sites.

1. Insert the sample reservoirs into vials.

2. Pipet 0.5 ml of a passivation solution (see *Preparation of Media and Reagents* for a list of suggested passivation solutions) into each sample reservoir. Cap and soak for at least 2 hours (or overnight) at room temperature.

3. Uncap and rinse all devices thoroughly with distilled water.

4. To remove any residual solution, add 0.5 ml of distilled water to each device, close the cap, and spin at 1,000 × *g*. Repeat.

5. To eliminate the remaining water, invert the sample reservoir in the vial, and then spin once more at 1,000 × *g* for 3 minutes.

6. If not using the devices immediately, add 100 µl of distilled water to the assembled sample reservoirs. Cap and store at 4°C.

Protein Concentration

Note *Use non-stick tubes during the concentration procedure to reduce loss of protein to absorption to the tube walls.*

1. Place the Microcon sample reservoir into a non-stick vial. Pipet the CBP-purified protein sample (i.e., the supernatant) into the sample reservoir. The maximum sample reservoir volume capacity is 0.5 ml. Avoid touching the membrane with the pipet tip. Close the cap.

2. Place the assembly into a microcentrifuge. Centrifuge the assembly at 14,000 × *g* for 50 minutes at 4°C or for 35 minutes at room temperature.

   Note *The centrifugation speed and duration given here are recommended for the Microcon YM-10 centrifugal filter unit and should be adjusted when using other devices.*

3. Following centrifugation, remove the assembly from the microcentrifuge and carefully remove the sample reservoir from the vial. Retain the sample reservoir.

4. Invert the sample reservoir and place the reservoir in a fresh non-stick vial. Close the cap.

5. Place the assembly into a microcentrifuge. Spin the assembly at 1000 × *g* for 3 minutes to transfer the concentrate to the vial.

6. Remove the assembly from the microcentrifuge and discard the sample reservoir. Retain the flow-through in the vial; this contains the concentrated proteins.

TROUBLESHOOTING

Adenovirus Production Troubleshooting

Observation	Suggestion
No colonies following transformation of BJ5183-AD-1 cells with shuttle vector	Perform a control transformation of the electroporation competent BJ5183-AD-1cells using the Transformation Control DNA to ensure that the cells are highly competent ($\geq 1 \times 10^7$ cfu/μg). Be sure to plate the transformations on kanamycin plates.
	If the concentration of linearized shuttle vector recovered from gel purification is too low, the volume of DNA solution used to transform the BJ5183-AD-1 cells may be increased to a maximum of 6 μl of DNA per 40 μl of cells.
All of the colonies following transformation of BJ5183-AD-1 cells are large (comparable to supercoiled DNA transformants)	Gel-purify the *Pme* I cut shuttle vector (containing the gene of interest) and ensure that the DNA is resuspended in sterile dH_2O prior to transformation.
Restriction digestion to confirm correct recombination produces bands of sizes other than 3.0, 4.5, and 30 kb following agarose gel electrophoresis	Provided the quality and yield of the miniprep DNA is adequate to make an evaluation, plasmids not producing bands of expected sizes following restriction digestion should be discarded. Choose additional well- isolated **small** colonies from the original plates and test by restriction digestion There are often faint background bands in BJ5183-AD-1 minipreps. If the PREDOMINANT bands are the expected sizes, redigest potential recombinants prepared from XL10-Gold cells. If after that second preparation there are still bands that are unaccounted for, discard the clone. For this reason it is recommended that more than one potential recombinant be amplified in XL10-Gold cells.
No colonies following transformation of XL10-Gold ultracompetent cells using recombinant Ad plasmid DNA	Perform a control transformation of the XL10-Gold cells using the pUC18 control DNA to ensure that the cells are competent ($\geq 5 \times 10^9$ cfu/μg). Be sure to plate the putative recombinant transformations on kanamycin plates.
No growth in liquid LB-kanamycin miniprep cultures	LB-kanamycin agar plates are weak or bad. Small colonies will grow alone or as satellites if the kanamycin plates are not at full strength. Prepare fresh LB-kanamycin agar plates using a fresh kanamycin stock solution, and do not add the kanamycin until the molten agar solution is ≤ 48°C.

TAP Purification Troubleshooting

Observation	Suggestion
The incidence of cell toxicity is high	Expression of the exogenous gene(s) may affect cell viability.
	When using cells expressing the SV40 large T antigen, the amount of SV40 origin-containing vector in the transfection reaction may need to be decreased by up to ten-fold.
Following elution from CBP resin, all expected proteins are not detected	Verify that the cell lysate contains the expected proteins before proceeding with the TAP purification. Protein expression must be detected by Western blotting using appropriate antibodies, following transduction and before performing the TAP purification.
	Determine if the proteins are present in the wash buffers to be discarded.
	Increase the salt concentration and/or detergent in the elution buffer(s). The salt concentration of each elution buffer provided is 150 mM NaCl.
Western analysis does not detect tagged protein(s)	Insert is cloned out of frame. Sequence to ensure correct reading frame. Reclone if insert is out of frame.
	Transfer of proteins is poor. Repeat transfer and optimize time of transfer, current and gel concentration and/or use molecular weight markers that cover the range to be transferred.
	Membrane preparation is inadequate. Ensure proper membrane hydration.
	Primary or secondary antibody concentration is too low. Titrate antibody conjugates for optimum concentrations.
	Protein has degraded during storage of the membrane. Use fresh blots.
	Proteolytic cleavage. Include protease inhibitors in lysis buffer.
The membrane produces excessive background	Insufficient blocking solution may have been used or the membrane was not thoroughly washed. Check the concentration of the blocking solution and/or wash thoroughly.
	Too much protein was loaded on gel. Load less protein on gel.
	Contamination by fingerprints and/or keratin has occurred. Use fresh membranes. Avoid touching the membrane. Use gloves and blunt forceps.
	The concentration of the secondary antibody is too high. Check the concentration of the antibodies and dilute if necessary.
	Do not add protease inhibitors to the CEB buffer.
Absence of peaks in mass spectrum	An insufficient amount of protein was used. Mass spectrometry requires at least 50 ng of protein. An adequate number of flasks must be processed to purify detectable amounts of protein. When characterizing the control purification products, 1×10^8 cells must be processed.
Too many peaks in the mass spectrum	The sample may be contaminated with keratin or other exogenous proteins. Wear gloves throughout the procedure and take care to use implements (razor blades, spatulas and glass plates) that have been freshly cleaned with ethanol.

PREPARATION OF MEDIA AND REAGENTS

LB Agar (per Liter)

10 g of NaCl
10 g of tryptone
5 g of yeast extract
20 g of agar
Add deionized H_2O to a final volume of 1 liter
Adjust pH to 7.0 with 5 N NaOH
Autoclave

LB Broth (per Liter)

10 g of NaCl
10 g of tryptone
5 g of yeast extract
Add deionized H_2O to a final volume of 1 liter
Adjust pH to 7.0 with 5 N NaOH
Autoclave

LB–Kanamycin Agar (per Liter)

Prepare 1 liter of LB agar
Autoclave
Cool to 55°C
Add 5 ml of 10-mg/ml filter-sterilized kanamycin
Pour into petri dishes (~25 ml/100-mm plate)

LB–Kanamycin Broth (per Liter)

Prepare 1 liter of LB broth
Autoclave
Cool to 55°C
Add 5 ml of 10-mg/ml filter-sterilized kanamycin

NZY+ Broth (per Liter)

10 g of NZ amine (casein hydrolysate)
5 g of yeast extract
5 g of NaCl
Add deionized H_2O to a final volume of 1 liter
Adjust to pH 7.5 using NaOH
Autoclave
Add the following filer-sterilized supplements prior to use:
12.5 ml of 1 M $MgCl_2$
12.5 ml of 1 M $MgSO_4$
20 ml of 20% (w/v) glucose (or 10 ml of 2 M glucose)

LB-Ampicillin Agar (per Liter)

10 g of NaCl
10 g of tryptone
5 g of yeast extract
20 g of agar
Adjust pH to 7.0 with 5 N NaOH
Add deionized H_2O to a final volume of 1 liter
Autoclave
Cool to 48°C
Add 10 ml of 10-mg/ml-filter-sterilized ampicillin
Pour into petri dishes (~25 ml/100-mm dish)

TE Buffer (1×)

10 mM Tris-HCl (pH 7.5)
1 mM EDTA
Autoclave

TE Buffer (0.1×)

100 µl TE Buffer (1×)
900 µl sterile dH20

1× TAE Buffer

40 mM Tris-acetate
1 mM EDTA

PBS

137 mM NaCl
2.6 mM KCl
10 mM Na_2HPO_4
1.8 mM KH_2PO_4
Adjust the pH to 7.4 with HCl

Growth Medium	Freezing Medium (100 ml)
DMEM (containing 4.5 g/L glucose and 110 mg/L sodium pyruvate and 4 mM L-glutamine), supplemented with 10% (v/v) heat-inactivated fetal bovine serum	50 ml DMEM (containing 4.5 g/L glucose, 110 mg/L sodium pyruvate and 4 mM L-glutamine) 40 ml heat-inactivated fetal bovine serum 10 ml dimethylsulfoxide (DMSO) Filter sterilize
Trypsin-EDTA Solution	**Suggested Passivation Solutions**
0.53 mM tetrasodium ethylenediamine-tetraacetic acid (EDTA) 0.05% trypsin	1% IgG in PBS 1% BSA in PBS 5% Tween® 20 in distilled water 1% powdered milk in distilled water 5% PEG compound in distilled water 5% Triton® X in distilled water 5% SDS in distilled water

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ENDNOTES

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MSDS INFORMATION

Material Safety Data Sheets (MSDSs) are provided online at *http://www.genomics.agilent.com*. MSDS documents are not included with product shipments.