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Effective modifications for improved homologous recombination and high-efficiency generation of recombinant adenovirus-based vectors

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Abstract

Generation of adenovirus-based vectors through homologous recombination within *Escherichia coli* cells is one of the most efficient strategies. A common challenge associated with this method is the formation of colonies containing self-ligated shuttle plasmid. To improve homologous recombination, a new pAdEasy-1-bearing competent cell line was constructed so that it no longer requires co-transformation with two plasmids and can generate more recombinant colonies (ninefold). New and efficient approaches were also tested to block shuttle plasmid self-ligation by a combined treatment of the plasmid with *Taq* DNA polymerase and calf intestine phosphatase (CIP) or blocking the formation of self-ligated plasmid-containing colonies by subcloning a suicide gene, *ccdB*, into the plasmid construct. Present experimental data show that these modifications are effective in eliminating self-ligated plasmid-containing colony background and offer greater simplicity, faster experimental progress, and higher efficiency in performing homologous recombination within *E. coli* cells, which could facilitate the production of high-titer infectious viral particles.

Keywords

Recombinant adenovirus-based vectors; Homologous recombination; Shuttle plasmid self-ligation; Transfection

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1. Introduction

Viruses are natural “professional” vehicles for transferring foreign nucleic acids into host cells, making them useful vectors for gene therapy (Kay et al., 2001). Recombinant adenoviruses received early attention as vehicles to transfer genes into the respiratory epithelium of patients being treated for diseases such as cystic fibrosis (Rosenfeld et al., 1992; Rich et al., 1993; Zabner et al., 1994; Johnson et al., 1995). Quickly it was realized that adenovirus-based vectors were a versatile gene delivery system, capable of highly efficient heterologous gene delivery into a broad range of cell types, both quiescent and dividing (Brody and Crystal, 1994; Kovacs et al., 1997; Hitt et al., 1997; Krasnykh et al., 1998; Benihoud et al., 1999; Kay et al., 2001). Adenovirus-based vectors do not integrate into the host cell genome, avoiding insertional mutagenesis within target cells. Capsid modifications of these vectors are produced easily, allowing retargeting of their tropism to different tissues (Dmitriev et al., 2002; Belousova et al., 2002; Gaden et al., 2004; Alba et al., 2005; Hesse et al., 2007). Furthermore, high-titered vector stocks can be prepared readily, which is a prerequisite for efficient *in vivo* gene delivery. Finally, new scale-up adenovirus production systems have been developed, facilitating their use in human clinical trials (Garnier et al., 1994; Côté et al., 1998; Lee et al., 2003; Cortin et al., 2004).

Decades of studying adenovirus biology have led to a clear understanding of the viral life cycle, genomic organization, and functions of the majority of viral proteins. Adenoviruses are medium-sized (80–100 nm), nonenveloped, icosahedral, complex, double-stranded DNA viruses that induce a cytopathic reproductive cycle within a large variety of somatic cells. To date, 51 immuno-logically distinct human adenovirus serotypes have been identified. Among them, serotypes 2 (Ad2) and 5 (Ad5) have been used extensively to construct replication-defective gene-transfer vectors for mammalian cells. The genetic information of Ad2 and Ad5 is compacted within linear, 36 kb long, double-stranded DNA genomes, with both strands coding for transcripts, nearly all of which are spliced heavily. Conventional adenoviral transcription units are referred to as early (E1–E4) and late, depending on their temporal expression to the onset of viral DNA replication (Horwitz, 1996; Shenk, 1996).

The high density and complexity of the adenoviral genome poses problems for recombinant manipulation, usually restricting manipulation to certain regions. In most recombinant adenoviral vectors, transgenes are introduced in place of E1 and E3. The E1 gene is essential for the assembly of infectious virus particles and is supplied *in trans* by packaging cell lines such as 293 (Graham et al., 1977) and 911 (Fallaux et al., 1996). Deletions within E1 render the virus defective in replication and production of infectious viral particles within target cells. The E3 gene encodes proteins involved in evading host immunity. Accordingly, vector viruses retain most of the viral genome within an approximately 30 kb DNA molecule (Crouzet et al., 1997; He et al., 1998).

Several approaches have been used to generate recombinant adenoviruses. The first method involves direct ligation of adenoviral DNA fragments to restriction endonuclease fragments containing the gene of interest (Ballay et al., 1985; Rosenfeld et al., 1991). *In vitro* manipulation is technically challenging due to the relatively large vector genome (36 kb), lack of suitable unique restriction sites and low efficiency of large DNA fragment ligation (Becker et al., 1994; Ketner et al., 1994). The second approach, which is used more commonly, involves cloning a gene of interest into a shuttle vector and then transferring the gene into the adenovirus genome by means of homologous recombination within an adenovirus packaging cell line (Ballay et al., 1985; Becker et al., 1994). The resulting desired recombinants are identified by screening individual plaques generated in a lawn of packaging cells (Becker et al., 1994). This approach has proven to be extremely useful, but the low efficiency of homologous recombination, the need for repeated rounds of plaque purification, and the long durations

required for completion of the viral production process have hampered the widespread use of adenovirus-based vectors.

More recently, a third approach has been developed that takes advantage of the highly efficient homologous recombination machinery present in *Escherichia coli* to generate recombinant adenovirus through a double-recombination step between a co-transformed adenoviral backbone plasmid vector and a shuttle plasmid vector carrying a gene of interest (Ketner et al., 1994; Chartier et al., 1996; Crouzet et al., 1997; He et al., 1998). This approach obviated the enzymatic manipulation and ligation steps involved in generating the adenoviral recombinants by previous methods. However, generation of self-ligated shuttle plasmids following recombination in *E. coli* cells produces undesirable to background colonies, which can lower the efficiency for selecting recombinant adenoviral plasmids. Thus, new approaches are needed to improve homologous recombination and selection for recombinant adenoviral colonies.

In this paper several new approaches were tested and evaluated to improve current conditions for preparing recombinant adenoviral vectors through homologous recombination. First, a modified strain of competent BJ5183 cells harboring adenoviral backbone DNA was generated to enhance homologous recombination through chemical transformation. Secondly, the linearized and dephosphorylated shuttle plasmid was treated with *Taq* DNA polymerase prior to homologous recombination within *E. coli* cells to ensure correct recombination. Lastly, a bacterial suicide gene, *ccdB* (Bernard and Couturier, 1992; Bernard et al., 1993, 1994), was subcloned into the backbone of the shuttle plasmid to prevent shuttle plasmid self-ligation. Experimental tests demonstrated that these modifications were effective in generating valid recombinant adenoviral plasmids with nearly zero self-ligated shuttle-plasmid background and producing high-titered infectious adenoviral vectors in 293T packaging cells.

2. Materials and methods

2.1. Plasmid construction

AdEasy™ Adenoviral Vector Systems were purchased from Stratagene (La Jolla, CA, Cat. No. 240009). To construct a shuttle plasmid that expressed GFP as a reporter, a GFP open reading frame (ORF) was amplified by polymerase chain reaction (PCR) from pcDNA3.1 CT-GFP (Invitrogen, Carlsbad, CA, Cat. No. 482001) with a primer pair of 5'-gagatatacgtcgaggctgacgagc-3' and 5'-gtaatacagactcactatag-3' in a 25-μL reaction volume. PCR product was cloned into a TA cloning vector pCR2.1 (Invitrogen, Carlsbad, CA, Cat. No. KNM2000-01) by following the manufacturer's instructions. The GFP gene was released subsequently from the resultant pCR2.1-GFP plasmid by digestion with *EcoR* V (NEB, Beverly, MA, Cat. No. R0195S) and then ligated into *EcoR* V-linearized pShuttle-CMV plasmid DNA. Insertion of GFP in pShuttle-CMV was verified by colony-PCR using primers 5'-gaagtgaatctgaataatttgc-3' and 5'-gtaatacagactcactatag-3' and DNA sequencing. The resultant pShuttle-CMV-GFP plasmid was transfected into 293T cells and GFP expression was evaluated using an inverted fluorescent microscope at 24 h post-transfection.

To construct a pShuttle-CMV-GFP-*ccdB* plasmid, a *ccdB* expression cassette was amplified by PCR from pSilent-R1/R2/*ccdB* (a kind gift from Dr. Shaobin Zhong, Department of Plant Pathology, North Dakota State University) with primers 5'-gctagtttaaacgacctgcagactggc-3' and 5'-gcatcaagaacagaagtatgc-3'. In this PCR amplification, Deep Vent DNA polymerase (NEB, Beverly, MA, Cat. No. M0258S) was used and the PCR product was directly ligated into pShuttle-CMV-GFP DNA that had been linearized with *Pme* I (NEB, Cat. No. R0560S). Ligates were then transformed into a *ccdB*-survival *E. coli* strain, DB3.1 (Invitrogen, Carlsbad, CA, Cat. No. 11782-018). Insertion of the *ccdB* gene was verified by PCR under the same conditions as its amplification and DNA sequencing. The killing of BJ5183 cells by *ccdB* gene

products was verified by transforming the plasmid DNA into BJ5183 competent cells. Transformations with the *ccdB*-containing plasmid clones were evaluated by colony formation.

2.2. DNA treatments by *Pme* I, calf intestine phosphatase (CIP) and *Taq* DNA polymerase

Approximately 100 µg of pShuttle-CMV-GFP DNA was linearized with *Pme* I and then separated by 0.8% agarose gel electrophoresis, followed by gel extraction (QIAquick Gel Extraction Kit, Qiagen, Valencia, CA, Cat. No. 28706). Recovered DNA was quantified by OD absorption using a UV spectrophotometer (Beckman DU 800). Approximately 20 µg of recovered DNA was dephosphorylated with CIP (NEB, Beverly, MA, Cat. No. M0290S) and then precipitated with EtOH and dissolved in 50 µL ddH₂O. Twenty-five microliters of dissolved DNA was included in a 50-µL PCR master mix with 1.0 unit *Taq* DNA polymerase, and incubated at 72 °C for 30 min. After incubation, treated DNA was precipitated with EtOH, dissolved in 25 µL ddH₂O and used for transformation of competent *E. coli* or self-ligation tests. One hundred nanograms of plasmid DNA treated as specified was included in a 10-µL ligation mix with 1 Unit T4 DNA ligase (Promega, Madison, WI, Cat. No. M1801) and incubated at 16 °C for 9 h. After self-ligation, 2 µL of the ligation mix was used to transform 200 µL plasmid-free BJ5183 competent cells.

2.3. Mammalian cell culture

293T cells were obtained from ATCC and grown with DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS) (HyClone, Logan, UT, Cat. No. SV30014.03), 100 U/mL penicillin, 100 µg/mL streptomycin sulfate, and 4 mM L-glutamine (Sigma-Aldrich St. Louis, MO, Cat. No. G1146) at 37 °C in a humidified 5.0% CO₂ incubator. Cells were split at a 1:6 ratio every 3–4 days.

2.4. Competent cell preparation and transformation

Highly-competent BJ5183 *E. coli* cells, with or without pAdEasy-1, were prepared according to a modified protocol as previously described (Hanahan, 1983). Treated bacteria cells were used immediately for transformation or kept at –80 °C for long-term storage by addition of 15% glycerol (Sigma-Aldrich, St. Louis, MO, Cat. No. G5516).

To transform competent cells with plasmid or ligation mix, 10–200 ng DNA was mixed with *E. coli* cells and incubated on ice for 30–60 min. Cells were then heat-shocked at 42 °C for exactly 90 s and then incubated on ice for 10 min. Eight hundred micro-liters of SOC was added to each heat-shocked tube and incubated at 37 °C for 60 min. Appropriate amounts of the transformation mixture were plated onto LB plates with the corresponding antibiotic for selection and incubated overnight. To test the competence of each preparation, 50 pg plasmid DNA was used per tube to avoid saturating the competent cells, followed by transformation as described. The transformation efficiency was calculated according to the following equation: colony forming units/µg DNA = [(# of colonies/pg DNA transformed) × (10⁶ pg/µg)]/% plated of total transformation.

2.5. Homologous recombination in *E. coli* cells

To carry out recombination, highly-competent BJ5183 cells carrying the pAdEasy-1 plasmid were prepared as described. pShuttle-CMV-GFP plasmid DNA was linearized by *Pme* I, dephosphorylated by CIP, and treated further by *Taq* DNA polymerase as described. For plasmid pShuttle-CMV-GFP-*ccdB*, DNA was either used in supercoiled form, or linearized by *Pme* I before transformation. After these treatments, 1.0 µg of treated shuttle plasmid DNA was added into each 200 µL aliquot of competent BJ5183 cells, transformed as described, plated on LB plates containing 50 mg/L kanamycin, and then incubated at 37 °C for 24–36 h. Well-isolated colonies were picked and inoculated in 3.0 mL LB medium with 50 mg/L

kanamycin for small-scale plasmid extraction. Extracted plasmids were dissolved in 200 μ l TE buffer, 5.0 μ l of which were used for primary identification through agarose gel electrophoresis. Self-ligated shuttle plasmid could be identified and discarded due to huge difference in molecular size between the shuttle plasmid and recombinant adenoviral plasmid. Recombinant adenoviral plasmids were further distinguished from pAdEasy-1 and stabilized by another round of transformation into a plasmid-stable *E. coli* strain (XL-10) and selection with 50 mg/L kanamycin.

2.6. Transfection into 293T cells and infectious adenoviral generation

Transfection of recombinant adenoviral DNA into 293T cells was performed with a dextran and polybrene enhanced calcium phosphate transfection method as described previously (Wu and Lu, 2007). Cultures were incubated at 37 °C in a 5.0% CO₂ incubator for 8 h and then media was replaced with 10 mL warm DMEM media with 10% FBS per flask. Conditioned medium was replaced with fresh medium every other day until 10–12 days post-transfection.

2.7. Viral stock preparation and amplification in 293T cells

To prepare primary viral-vector stocks, growth medium was discarded from transfected cells at day 10–12 post-transfection, leaving approximately 2.0 mL per flask. Flasks were subjected to four rounds of freeze–thaw cycles between an –80 °C freezer and a 37 °C water bath. At the last cycle, the cells were collected into a 15-mL centrifuge tube and centrifuged at 5000 rpm for 15 min. The supernatant was collected and aliquots of 0.5 mL/tube were stored at –80 °C. Titers of the viral stock were determined as described in the following “vector titration” section. To amplify titers of the primary viral stock, 293T cells were seeded 24 h before infection, allowing the formation of an approximately 70% cell monolayer the next day. At the time of infection, the medium was discarded and the cell monolayer was infected with 0.5 mL primary viral stock at 37 °C for 1.0 h. After infection, cells were washed twice with PBS and maintained in DMEM supplemented with 5% FBS. Infection of 293T cells and amplification of the vector virus was monitored by GFP expression using an inverted fluorescent microscope. Infected 293T cells were harvested when maximum cytopathic effect (CPE) appeared and then viral stocks were prepared as described above.

2.8. Vector titration

Titration of viral vectors generated in transfected 293T cells was performed by a limiting dilution method described previously (Wu and Lu, 2007). Infected cultures were kept at 37 °C in a CO₂ incubator for 3 days. Vector titers were determined by counting the number of GFP-positive cells at the end-point dilution as follows: Vector titer (IU/mL) = # of GFP⁺ cells $\times$ 5 (IF) $\times$ DF, where IU = infectious unit, IF = inoculum factor, and DF = dilution factor.

3. Results

3.1. Blocking shuttle vector self-ligation by Taq DNA polymerase treatment

As shown in Table 1, transformation of *E. coli* cells with ligation mix containing *Pme* I-linearized self-ligated plasmid DNA generated more than 1000 colonies. Additional CIP or *Taq* DNA polymerase treatments lowered the number of colonies to less than 10. It was notable that when both CIP and *Taq* DNA polymerase treatments were combined, no kanamycin-resistant colonies were observed. When treated DNA was used for recombinant adenovirus generation in *E. coli* cells, CIP-based treatment resulted in up to 60% of the colonies containing self-ligated shuttle plasmid DNA (Fig. 1A), while the combined treatments of CIP and *Taq* DNA polymerase induced no colonies with self-ligated shuttle plasmid DNA (Fig. 1B).

3.2. Homologous recombination in chemically competent BJ5183 *E. coli* cells carrying pAdEasy-1

To simplify homologous recombination, a new strain of BJ5183 *E. coli* strain harboring pAdEasy-1 was constructed. These newly generated BJ5183 *E. coli* cells possessed similar transformation competence to plasmid-free cells. As shown in Fig. 2, transformation of these cells with 1 μ g of pCR2.1 resulted in the formation of 4.9×10^7 and 5.8×10^7 kanamycin-resistant colonies on LB plates, respectively. In addition, transformation of these two types of competent cells with pShuttle-CMV-GFP plasmid DNA generated zero kanamycin-resistant colonies for plasmid-free competent cells as compared to 5.9×10^3 for the pAdEasy-1-carrying competent cells. Furthermore, co-transformation of these two competent cells with both pAdEasy-1 and pShuttle-CMV-GFP plasmid DNA resulted in nine times more colonies in pAdEasy-1-carrying competent cells than the control plasmid-free counterpart (2400 vs. 260). These experimental data indicate that the generation of pAdEasy-1-carrying competent cells no longer requires co-transformation of the shuttle plasmid and pAdEasy-1, thus, simplifying the transformation procedure, as well as largely enhancing the transformation efficiency.

3.3. Adaptation of the *ccdB* gene into the shuttle vector backbone

To block shuttle plasmid self-ligation, a *ccdB* expression cassette was incorporated into the *Pme* I restriction site of the pShuttle-CMV-GFP plasmid (Fig. 3A). As constructed, the resultant *ccdB*-carrying shuttle plasmid regained the *Pme* I restriction site, and thus, could still be linearized by *Pme* I in the same manner as the unmodified shuttle plasmid. The *ccdB* expression cassette was subcloned into the shuttle plasmid in such a way that it will be maintained if the shuttle plasmid DNA is self-ligated, but lost if a double-arm homologous recombination takes place between the two homologous arms of the shuttle plasmid and pAdEasy-1 (Fig. 3B).

To test if the incorporation of the *ccdB* gene into the shuttle plasmid would prevent the formation of BJ5183 cell colonies carrying self-ligated shuttle plasmid DNA, two types of shuttle plasmids were used to transform plasmid-free competent BJ5183 cells. As expected, transformation with the pShuttle-CMV-GFP plasmid into BJ5183 competent cells generated 6.8×10^7 kanamycin-resistant colonies from 1 μ g of parental plasmid DNA, while transformation with the *ccdB*-carrying shuttle plasmid did not generate any kanamycin-resistant self-ligated colonies (Fig. 4). The incorporation of the *ccdB* gene expression cassette completely blocks the formation of self-ligated colonies in BJ5183 *E. coli* cells.

3.4. Homologous recombination using the *ccdB* shuttle vector plasmid

The shuttle plasmid DNA was prepared and used to transform competent BJ5183 cells carrying the pAdEasy-1 plasmid. As shown in Fig. 5A, transformation with *Pme* I-linearized shuttle vector DNA generated an average of 2780 kanamycin-resistant colonies, while transformation with supercoiled shuttle vector DNA generated an average of 412 kanamycin-resistant colonies. It was notable that transformation with supercoiled shuttle vector DNA also generated quite a lot of kanamycin-resistant colonies (Fig. 5A). To ensure that these are *ccdB*-negative colonies, a total of 200 transformed-cell colonies were tested by PCR for their retention of the *ccdB* gene as an indication of shuttle plasmid self-ligation. None of these colonies contained the *ccdB* gene expression cassette (Fig. 5B). Further verification by agarose gel electrophoresis showed no shuttle plasmid band, which is characteristic of self-ligated shuttle plasmids (Fig. 5C). These results indicate that all surviving colonies are generated by recombinant adenoviral plasmids and do not contain any self-ligated shuttle plasmid DNA.

3.5. Verification of recombinant plasmids

After primary identification by agarose gel electrophoresis, plasmid DNA from representative colonies were transformed into *E. coli* strain XL-10, and selected as described. Plasmid DNA

derived from individual kanamycin-resistant colonies, along with control DNA from pAdEasy-1, pShuttle-CMV-GFP and pShuttle-CMV-GFP-*ccdB*, were digested with *Pac* I and analyzed by agarose gel electrophoresis. As shown in Fig. 6A, recombinant plasmids obtained through this optimized system showed the correct patterns of restriction bands. Moreover, there was no difference among recombinant plasmids derived from the *ccdB* gene-adapted shuttle plasmid and those from the unmodified plasmids. This was further confirmed by DNA transfection and production of infectious viral particles using 293T packaging cells as described.

3.6. Adenoviral DNA transfection and vector production

Pac I-digested recombinant plasmid DNA was transfected into 293T cells and robust GFP expression was observed within 24 h. Viral vector production continued to increase for 3–4 days post-transfection. Beyond this time period, GFP expression appeared to decrease in most of the transfected cells. However, in some transfected cells, infectious adenovirus particles were generated and able to infect and replicate within adjacent cells. This process continued and eventually led to the formation of infection foci within the transfected 293T cell monolayer 5–10 days post-transfection (Fig. 6B). This appeared to be a typical phenomenon documented by others in literature (He et al., 1998).

This study also confirmed that adenoviral-vector titers from the initial transfection could be significantly increased by repeated viral infection of fresh 293T cells. As shown in Fig. 6C, 100% GFP-positive cells were obtained on day 2 post-infection by infecting a 50% confluent 293T cell monolayer at an MOI of 10. In addition to GFP expression, infected 293T cells also showed the characteristic CPE associated with adenovirus infection (cell roundup). Following several rounds of consecutive infection and amplification, high-titer vector stocks up to 4.5×10^8 IU/mL were prepared without vector concentration (Fig. 6D).

4. Discussion

In this study, several attempts were taken to address the problems related to the self-ligation of shuttle plasmid, as well as the low recombination efficiency, within *E. coli* cells, through an initial transformation of BJ5183 cells with pAdEasy-1, growth selection with ampicillin, and preparation of chemically competent *E. coli* cells carrying the pAdEasy-1 plasmid. As a result, recombinant adenoviral plasmids were generated with high efficiency and zero background. The competence of the newly constructed pAdEasy-1-carrying cells was determined to be comparable to that of the plasmid-free counterpart cells. Instead of co-transformation with two plasmids, the plasmid-carrying competent cells mediated efficient generation of recombinant adenoviral plasmids by a single plasmid transformation. It should be noted that by homologous recombination within *E. coli* cells carrying the pAdEasy-1 plasmid, kanamycin-resistant colonies carry actually both the recombinant adenoviral plasmid and pAdEasy-1. This potential problem for preparing pure target plasmids can be solved by transforming the plasmid mix into another plasmid-stabilizing *E. coli* strain, such as DH5 α or XL-10, and selecting with kanamycin. New kanamycin-resistant colonies will carry only the recombinant adenoviral plasmid.

To address the issue of self-ligated shuttle plasmid production when preparing recombinant adenoviral plasmids, current approaches include linearization and dephosphorylation of the shuttle plasmid prior to co-transformation with pAdEasy-1. However, these treatments are not sufficient enough to block self-ligation. Experimental results show that the percentage of colonies carrying self-ligated shuttle plasmids can reach as high as 60% (Fig. 1). To promote more effective blockage of plasmid self-ligation, an additional treatment of shuttle plasmid DNA with *Taq* DNA polymerase was introduced following linearization and dephosphorylation. Experimental data obtained demonstrated that the additional *Taq* DNA

treatment blocked the formation of self-ligated shuttle plasmid completely. Through these treatments, the terminal transferase activity of *Taq* DNA polymerases is known to add a 3'-A overhang to each end of the DNA fragment (Clark, 1988), while CIP treatment creates ends that do not possess a 5' phosphate group. Thus, this combined treatment creates a condition that makes the self-ligation of shuttle plasmid DNA essentially impossible. *Taq* DNA polymerase mediated addition of a 3'-A overhang to linearized DNA ends is a well-documented molecular biology technique that has been very useful and widely employed in TA vector-based cloning and sequencing. In addition, this step of *Taq* DNA treatment was determined to cause no adverse impact on transformation. When the treated shuttle plasmid DNA was used to transform pAdEasy-1-carrying competent cells or co-transform plasmid-free competent cells with pAdEasy-1 DNA, over 5.9×10^3 and 2.6×10^2 recombinant colonies were generated, respectively. These data also argue for improved transformation efficiencies of competent cells carrying pAdEasy-1 for recombinant adenoviral plasmid generation.

In addition, another strategy was tested for knocking out shuttle plasmid self-ligation background by introducing a *ccdB* expression cassette between the two homologous recombination arms of the shuttle plasmid. Since *ccdB* was positioned this way, it will be lost upon successful recombination between the shuttle plasmid and pAdEasy-1, allowing colony formation of *E. coli* carrying the desired recombinant plasmid. Self-ligated shuttle plasmids will retain an intact *ccdB* gene, and the accumulation of *CcdB* protein will eventually kill *E. coli* cells and prevent them from forming colonies on LB plates. As demonstrated, transformation with pShuttle-CMV-GFP generated 6.8×10^7 colonies per microgram plasmid, while the *ccdB* containing plasmid, pShuttle-CMV-GFP-*ccdB*, did not generate any colonies. Furthermore, it was confirmed that transformation with shuttle plasmid DNA containing the *ccdB* expression cassette led to high efficiency generation of colonies carrying the desired recombinant plasmid. These findings together indicate that the *ccdB* gene can be used effectively for eliminating shuttle plasmid self-ligation in BJ5183 cells. Another advantage of using the *ccdB* gene to block shuttle plasmid self-ligation is that it works when transformed in supercoiled form. Theoretically, this holds an important implication because it broadens the range of heterologous genes that can be cloned into adenoviral vectors. The original system is dependent on the linearization of shuttle plasmid DNA carrying target gene by *Pme* I to reduce background. As a consequence, any gene sequence that carries *Pme* I restriction sites will not be introduced into recombinant adenoviral plasmids since it will be disrupted by *Pme* I digestion. It should be noted that no change has been introduced to the homologous arm sequences. Therefore, the described modifications to the adenoviral shuttle construct will not introduce any sequence mismatching during the adenoviral homologous recombination event.

In summary, these new modifications make the generation of recombinant adenoviral vectors easy, straightforward, efficient, and most importantly, reduces the background from self-ligated shuttle vector plasmid-containing colonies to zero. These modifications have proven to be efficient and rapid in creating recombinant adenoviral vectors expressing heterologous genes, and would be especially valuable for creating a large number of recombinant adenoviral vectors with different genes. These modifications will have significant implications in using adenovirus-based vectors for gene therapy research and clinical applications.

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Fig. 1.

Generation of recombinant adenoviral plasmids in bacterial cells. (A) DNA from pShuttle-CMV-GFP was linearized by *Pme* I, dephosphorylated, and used for transformation of competent BJ5183 *E. coli* cells. Plasmid DNA prepared from inoculated colonies, as described in Section 2, were analyzed in supercoiled form by 0.4% agarose gel electrophoresis and ethidium bromide staining. Lane M, Fisher 1 kb DNA ladder (Cat. No. BP2553-100); lanes 1–38, plasmid DNA from different colonies. Based on the difference in migration rates, colonies in lanes 2, 3, 5, 6, 9–12, 14, 15, 17, 18, 22, 24–26, 28–30, 32, 33, 35, 37 contained self-ligated shuttle plasmid DNA; the remaining 15 out of 38 colonies potentially contained valid recombinant aden-oviral plasmids. (B) Analysis of plasmid DNA from post-recombination colonies. In addition to the treatments in (A), pShuttle-CMV-GFP DNA was treated with Taq DNA polymerase as described in Section 2. Based on the migration rates, none of the 38 colonies contained self-ligated shuttle plasmid DNA.

Fig. 2.

Comparison of transformation and recombination efficiencies of competent cells made from *E. coli* BJ5183 and BJ5183 carrying pAdEasy-1. The competence of two types of cells was compared by transformation with 25 ng pCR2.1 plasmid and plating on kanamycin LB plates. The number of colonies was calculated as described in Section 2. Recombination efficiencies were compared by either transforming pAdEasy-1-carrying competent BJ5183 *E. coli* cells with 1.0 μg *Pme* I-linearized, CIP and Taq DNA polymerase treated pShuttle-CMV-GFP plasmid DNA alone, or co-transforming plasmid-free competent cells with 500 ng of treated pShuttle-CMV-GFP and 500 ng of supercoiled pAdEasy-1 plasmid DNA. Numbers of colonies were counted as described. Data was obtained from representative experiments performed in triplicate.

Fig. 3. *CcdB* gene adaptation to the adenovirus vector system and functional testing. (A) Schematic map of the *ccdB* gene cloned into shuttle-CMV-GFP. *CcdB* gene was inserted between the left and right homologous recombination arms of the shuttle vector plasmid so that it will be lost upon recombination, while if self-ligation occurs, it will block colony formation by eventually killing BJ5183 cells. (B) Functional test of cloned *ccdB* gene on BJ5183 cells. 25 ng of both pShuttle-CMV-GFP and pShuttle-CMV-GFP-*ccdB* plasmids were used to transform 200 μL of competent BJ5183 cells. The number of colonies formed per microgram of each plasmid DNA was determined as described in Section 2. Data was obtained from representative experiments performed in triplicate.

Fig. 4.

Schematic outline of the optimized recombinant adenovirus vector system through plasmid recombination in *E. coli* cells. First, the gene of interest is cloned into a shuttle vector, such as the illustrated pShuttle-CMV-GFP-*ccdB* as illustrated; Second, the resulting plasmid is digested with a restriction enzyme, *Pme* I (*ccdB*-containing plasmids can also be used in supercoiled form). For shuttle plasmids not containing the *ccdB* gene, linearized DNA is further treated with CIP and *Taq* DNA polymerase; Third, competent BJ5183 *E. coli* cells carrying pAdEasy-1 is transformed with linearized plasmid DNA; Fourth, the recombinant adenoviral plasmid is digested with *Pac* I to expose the adenoviral inverted terminal repeats (ITR) and transfected into a 293T packaging cell line. Infectious recombinant adenoviral vector particles are obtained from the transfected cells and amplified by infecting fresh 293T cells.

Fig. 5.

Generation of recombinant adenovirus plasmids using the *ccdB*-containing shuttle plasmid. (A) Competent BJ5183 *E. coli* cells were transformed using 1.0 μ g *Pme* I-linearized DNA from pShuttle-CMV-GFP-*ccdB* plasmid, or supercoiled DNA from of the same plasmid, and numbers of recombinant colonies were counted as described in Section 2. Data was obtained from representative experiments performed in triplicate. (B) Detection of shuttle plasmid self-ligation by PCR using primers specific for the amplification of the *ccdB* gene cassette. Two hundred colonies transformed with linearized and supercoiled shuttle plasmid DNA were tested and none of them were found to contain the *ccdB* gene. (C) Detection for shuttle plasmid self-ligation by agarose gel migration. As in Fig. 1(A and B), none of the colonies contained self-ligated shuttle plasmid DNA based on the migration rates. Lane M, Fisher λ DNA-Hind III digest DNA ladder (Cat. No. BP2556-200); lane C1, control plasmid DNA from pAdEasy-1; lane C2, control plasmid DNA from pShuttle-CMV-GFP-*ccdB*; lanes 1–17, plasmid DNA from colonies generated by transforming *Pme* I linearized pShuttle-CMV-GFP-*ccdB* DNA; lanes 18–34, plasmid DNA from colonies generated by transforming supercoiled pShuttle-CMV-GFP-*ccdB* DNA.

Fig. 6. Recombinant adenoviral plasmid transfection and infectious virus generation in HEK 293T cells. (A) Representative *Pac* I digestion of DNA from plasmids pShuttle-CMV-GFP, pShuttle-CMV-GFP-*ccdB*, pAdEasy-1, and recombinant adenoviral plasmids from recombination using DNA from either pShuttle-CMV-GFP or pShuttle-CMV-GFP-*ccdB*. Lane M1, Fisher 1 kb DNA ladder; lane M2, Fisher λ DNA-Hind III digest DNA ladder; lane 1, pShuttle-CMV-GFP; lane 2, pShuttle-CMV-GFP-*ccdB*; lane 3, pAdEasy-1; lane 4, recombinant adenoviral plasmid from recombination using DNA from pShuttle-CMV-GFP; lane 5, recombinant adenoviral plasmid from recombination using DNA from pShuttle-CMV-GFP-*ccdB*. Digested DNA was analyzed by 0.4% agarose gel electrophoresis. Based on the migrations, characteristic bands

of correct size were observed for each plasmid, and the recombinant adenoviral plasmids derived from each shuttle plasmid showed the same characteristic bands (a 4.5-kb band from bacterial plasmid backbone and a 31.8-kb band from recombinant adenoviral DNA). (B) DNA from recombinant adenoviral plasmids were digested with *Pac* I, precipitated with EtOH, and transfected into 293T cells as described in Section 2. Representative photomicrographs taken on day 10 post-transfection demonstrate the formation of infection loci within the transfected cell monolayer. (C) Primary viral stock was amplified by infecting fresh 293T cell monolayer. A 50% confluent cell monolayer was infected at an MOI of 10 and representative photomicrographs were taken on day 2 post-infection. (D) Consecutive amplification of viral stock in 293T cells. When the stock titers were relatively low (less than 10^8 IU/mL), each amplification process gradually boosted the titer about 10 fold. Data was obtained from representative experiments performed in triplicate. FL, fluorescent light; NL, normal light.

Table 1

Effect of selected treatments on colony formation of transformed competent BJ5183 cells with self-ligated shuttle vector plasmid DNA

Plasmid DNA treatment	Number of self-ligated colonies
<i>Pme</i> I	$1.3 \pm 0.4 \times 10^3$
<i>Pme</i> I + CIP	7.25 ± 2.5
<i>Pme</i> I + <i>Taq</i>	2.0 ± 1.4
<i>Pme</i> I + CIP + <i>Taq</i>	0.0

Pme I: shuttle vector plasmid DNA was linearized by *Pme* I; CIP: shuttle vector plasmid DNA was dephosphorylated by calf intestinal phosphatase (CIP); *Taq*: shuttle vector plasmid DNA was treated with one unit of *Taq* DNA polymerase at 72 °C for 30 min. 100 ng of each treated plasmid DNA was used in a ligation test and transformed into plasmid-free competent BJ5183 cells. Transformed cells were plated on LB plates with 50 mg/L kanamycin. The numbers of colonies formed were calculated.