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A simple negative selection method to identify adenovirus recombinants using colony PCR

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ABSTRACT

Background: The AdEasy system is a fast-track system for generating recombinant adenoviruses using the efficient homologous recombination machinery between shuttle and adenovirus backbone plasmids in *Escherichia coli* BJ5183 cells. The key step is homologous recombination in BJ5183 cells, which is driven by RecA activity. However, culture time is stringently limited to reduce the damage to recombinant plasmids by RecA activity. Therefore, rapid identification of recombinant adenoviruses within the limited time-period is critical.
Results: We developed a simple negative selection method to identify recombinant adenoviruses using colony PCR, which improves the efficiency of adenovirus recombination screening and packaging.
Conclusions: The negative selection method to identify AdEasy adenovirus recombinants by colony PCR can identify the recombined colony within a short time-period, and maximally avoid damage to the recombinant plasmid by limiting recombination time, resulting in improved adenovirus packaging.

1. Introduction

Adenoviral vectors are a versatile tool to investigate gene expression and regulation as well as gene therapy. Several advantages of adenoviral vectors have been demonstrated [1,2], which include the inability to integrate into the genome of target cells, broad spectrum application to various cell types, high recombinant gene expression, the ability to produce high titers of recombinant virus, and the ability to transfer genes independent of active cell division.

Thus far, numerous approaches have been developed to generate recombinant adenoviruses. The AdEasy system involves homologous recombination in *Escherichia coli* BJ5183, which provides a strategy that simplifies the generation and production of adenoviruses [3]. A recombinant adenoviral plasmid is generated with minimal enzymatic manipulations and uses homologous recombination in bacteria rather than eukaryotic cells. This system may expedite the process of generating and testing recombinant adenoviruses for various applications [4].

The AdEasy system is based on the ability of RecA protein in *E. coli* BJ5183 to pair an oligonucleotide to its homologous sequence in duplex DNA and to form a three-stranded complex, and then complete homologous recombination. Thus, RecA plays a central role in homologous recombination [5,6]. BJ5183 cells show a relatively high frequency of homologous recombination, while unwanted or detrimental rearrangements and/or recombinations of large recombinant plasmids in BJ5183 cells can occur. RecA activity is required to achieve homologous recombination between a linearized shuttle plasmid and supercoiled adenovirus backbone plasmid, but also damages the recombined backbone plasmid [7]. Therefore, the culture time for adenovirus recombination in BJ5183 cells must be stringently controlled to reduce damage. After recombination is confirmed, culture is stopped and the plasmid extracted. However, traditional adenovirus recombination indentified by PacI digestion is time-consuming, labor-intensive, and difficult to limit culture time. To solve low efficiency and the difficulty to screen homologous recombination, and rapidly identify adenovirus recombinants within a limited time-period, we developed a negative selection method to identify adenovirus recombinants using colony PCR, which improves the efficiency of adenovirus recombination screening and packaging.

2. Materials and methods

2.1. Cloning of target genes into the pAdTrack-CMV shuttle vector

Recombinant hVEGF165/pTA2 plasmid containing human VEGF165 intact ORF (M32977) and shuttle plasmid pAdTrack-CMV were digested with KpnI and XbaI (TOYOBO, Japan). Recombinant hMCL1L/pTA2 plasmid containing human MCL1L intact ORF (AF118124) and shuttle plasmid pAdTrack-CMV were digested with SalI (TOYOBO, Japan) and

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Table 1
Primer sequences for colony PCR.

Designation	Primer sequence	Tm	Amplicon size (bp)
hVEGF165 PCR (for M32977)	P1: (F)5′-GAAACCATGAACTTTCTGCTGTCTTG-3′ P2: (R)5′-CTCACCGCCTCGGCTTGTCA-3′	68 °C	583
hMCL1L PCR (for AF118124)	P3: (F) 5′-GCGGCGACTGGCAATGTTTG-3′ P4: (R)5′-AGTTACAGCTTGGAGTCCAACTGCA-3′	68 °C	1152
pAdTrack-CMV PCR	P5: (F)5′-CTTTCGCTGTACGGCAGTAGTCG-3′ P6: (R)5′-CCTATGGGGGCTGTAATGTTGTCTCTA-3′	68 °C	1002

XbaI. After digestion, hVEGF165 and hMCL1L cDNAs and the linearized pAdTrack-CMV vector were recovered using an Agarose Gel DNA Fragment Recovery Kit (Sangon, China), and then ligated together using T4 DNA ligase followed by transformation into high competent *E. coli* DH10B. Transformed positive clones for hVEGF165 and hMCL1L/pAdTrack-CMV plasmids were screened by routine colony PCR using P1 and P2, and P3 and P4 primers (Table 1), and verified by dual restriction enzyme digestion. PCR conditions were as follows: 98°C for 6 min, 35 cycles at 98°C for 20 s and 68°C for 1 min, followed by 72°C for 10 min. PCR product sizes for hVEGF165 and hMCL1L were 583 bp and 1152 bp, respectively.

2.2. Generation of recombinant adenovirus plasmids in BJ5183 cells

Recombinant shuttle vector pAdTrack-CMV (2 μg) containing the target gene was linearized with PmeI (NEB, USA), and 5 μl digested product was transformed into high competent AdEasier cells using the calcium chloride transformation method [8] for homologous recombination with the adenovirus backbone plasmid. Culture time on the lysogeny broth (LB) agar plates containing 50 μg/ml kanamycin was limited to 16–20 h [7].

2.3. Identification of recombinants by colony PCR

The PCR mixture was prepared as follows: 10 μl 2× Taq Plus PCR Master Mix (Tiangen, China), 1 μl P5 (10 μM) and 1 μl P6 (10 μM) primers (Table 1) and 8 μl ddH_2O. The positive plasmid group used the circular shuttle vector pAdTrack-CMV as a template and was compared with that of the negative plasmid group (without template). A needle was used to pick six of the smallest needlepoint-like colonies from individual LB agar plate, which were added to the PCR mixture, and chosen by colony PCR screening, meanwhile these six colonies were individually cultured in 5 ml LB medium containing 50 μg/ml kanamycin for 10 h in a 37°C orbital shaker, and performed the corresponding markers. PCR conditions were as follows: 98°C for 6 min, 35 cycles at 98°C for 20 s and 68°C for 1 min, followed by 72°C for 10 min. After culturing for 10 h, cells were collected and plasmid DNA was purified using a plasmid mini preparation kit (Sangon, China) according to the manufacturer's instructions. The negative recombinant clones screened from colony PCR were reserved for further confirmation.

2.4. Identification by PacI digestion

Purified recombinants were transformed into high competent *E. coli* DH10B. One clone was picked from each transformed LB agar plate and cultured in 5 ml LB medium containing 50 μg/ml kanamycin overnight in a 37°C orbital shaker. A small amount of recombinant plasmid that was amplified in DH10B cells was prepared for PacI digestion. Also, PCR screening of the target gene was performed for comparison with the previous colony PCR screening.

2.5. Generation of recombinant adenoviruses in 293FT cells

Six microgram of purified recombinant adenovirus plasmid hVEGF165 was prepared by a balanced mass mixture with recombinant clones 1, 3–6, and hMCL1L by clones 8–10, followed by digestion with PacI to liberate both inverted terminal repeats (ITRs), and then purified with ethanol precipitation. Packaging 293FT cells were plated on 6 well plates and transfected with 2 μg PacI-digested purified sterile plasmid DNA using Lipofectamine 2000 (Invitrogen, USA), according to the manufacturer's instructions. Viral production was observed 7–10 d after transfection. Cells were then collected, repeatedly freeze–thawed, and 50 μl supernatant was used to infect COS-7 (African Green Monkey SV40-transfected kidney fibroblast cell line; ATCC, USA) cells. After 72 h, cells were collected and protein extracted for Western blot detection of the target gene product for comparison with that of the non-infected group and empty adenovirus infected group.

Proteins were isolated in a radioimmunoprecipitation assay buffer containing a protease inhibitor cocktail and phosphatase inhibitors (R0278; Sigma, USA). SDS-PAGE was performed in a 12% polyacrylamide gel, and proteins were transferred onto a polyvinylidene fluoride membrane in transfer buffer for 1 h using a Bio-Rad Semi-Dry apparatus. Washes and incubations were performed using standard procedures. Anti-human VEGF165 (cat 07-1419, 1:1000) and anti-human MCL1 polyclonal antibodies (cat AB2910, 1:2000; Millipore, USA) were used as primary antibodies. The secondary antibody was a horseradish peroxidase (HRP)-labeled anti-mouse-IgG (1:2000; Zhongshan Bio-tech,

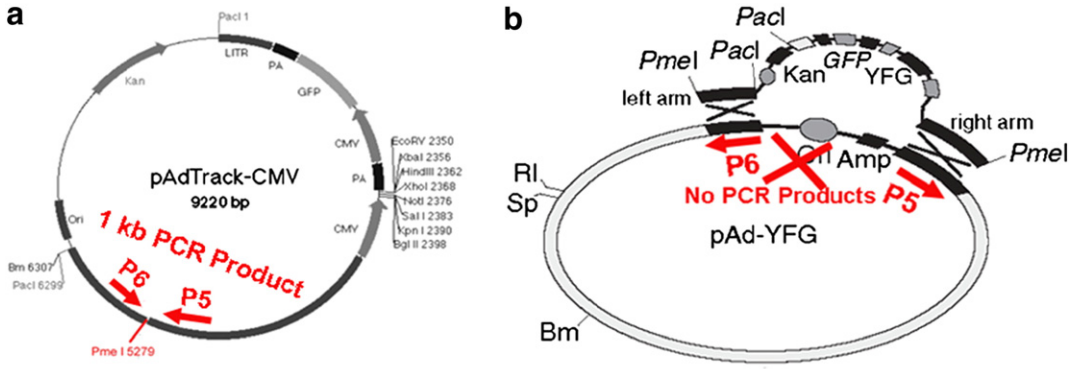

Fig. 1. Strategy of the negative selection method to identify adenovirus recombinants using colony PCR. (a) P5 and P6 primers amplify an approximately 1 kb product from the circular pAdTrack-CMV shuttle plasmid by PCR. (b) After recombination of shuttle and pAdEasy adenovirus backbone plasmids, the amplified area of the primer extends an approximately 30 kb adenovirus backbone sequence. However, commonly used Taq polymerase does not efficiently amplify the long sequence. PCR products of the recombinant are negative and non-recombination of the shuttle vector is positive.

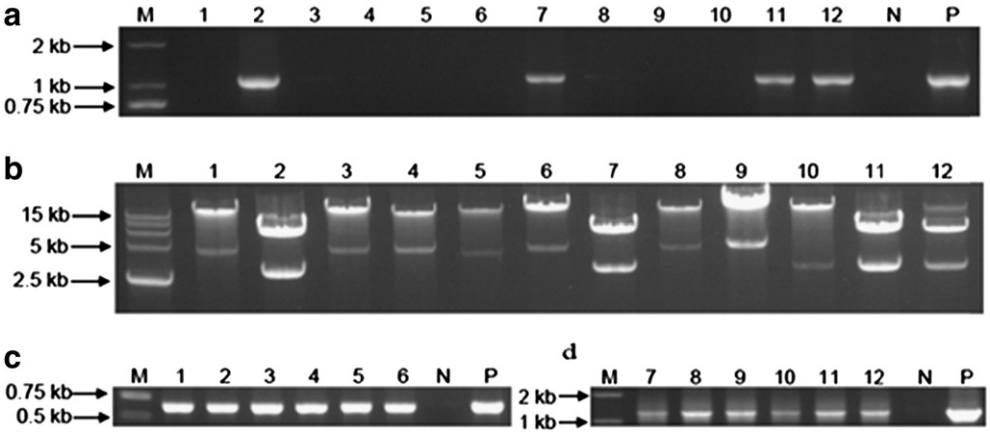

Fig. 2. Identification of recombinant adenoviral vectors by colony PCR. (a) Colony PCR screening of the six smallest randomly-picked clones per target gene. All except four (clone nos. 2, 7, 11 and 12) of the recombinant clones are potential recombinants because a specific 1 kb product is absent. (b) PacI restriction endonuclease digestion of candidate recombinants. All of the twelve tested clones from (a) were validated by this analysis. Seven of the twelve clones released a 4.5 kb fragment after PacI digestion, one released a 3 kb fragment, and the other four did not release a fragment larger than 15 kb. (c) PCR identification of hVEGF165 adenovirus recombinants. All six clones show a specific 583 bp band. (d) PCR identification of hMCL1 adenovirus recombinants. All six clones show a specific 1152 bp band. M: DNA markers, Lanes 1–6: Samples hVEGF165 adenoviruses recombinant, Lanes 7–12: Samples hMCL1 adenovirus recombinants, Lane P: Positive control, Lane N: Negative control.

China). An HRP-labeled anti-GAPDH monoclonal antibody (Clone KC-5G5; 1:5000; KangChen Bio-Tech, China) was used to detect GAPDH. Western blots were visualized using an ECL reagent (Pierce Biotechnology, USA) and a Storm 860 PhosphorImager.

3. Results and discussion

Adenoviral vectors have been widely used as efficient gene delivery vehicles for gene therapy and vaccine development, as well as for gene function studies. The AdEasy system is a simple and efficient method for rapid generation of recombinant adenoviruses. The recombination step is the most critical and performed in *E. coli* BJ5183 rather than mammalian cells, which uses the high homologous recombination activity of RecA in bacteria [3]. RecA activity is a double-edged sword, which is required for homologous recombination between linearized shuttle and supercoiled adenovirus backbone plasmids, while the least amount of damage to the recombined backbone plasmid is desired. Unwanted or detrimental rearrangements and/or recombinations of large recombinant plasmids can occur in BJ5183 cells [6,7]. Therefore, we optimized the culture time of adenovirus recombinants in BJ5183 cells after homologous recombination was completed. The traditional way to identify adenovirus recombinants via routine PacI digestion is very labor-intensive and time consuming. To improve the screening efficiency of homologous recombination, and quickly identify adenovirus recombinants within a limited time-period, we developed a negative selection method to identify adenovirus recombinants using colony PCR, which improves the efficiency of adenovirus recombination screening and packaging.

First, we designed a primer pair across the PmeI restriction enzyme site in the shuttle plasmid, which is commonly used for linearization. P5 and P6 primers were located in the left and right homologous recombination arms, respectively. If the template is a circular shuttle plasmid, the primer pair amplifies an approximately 1 kb product. After homologous recombination is achieved between the shuttle and adenovirus

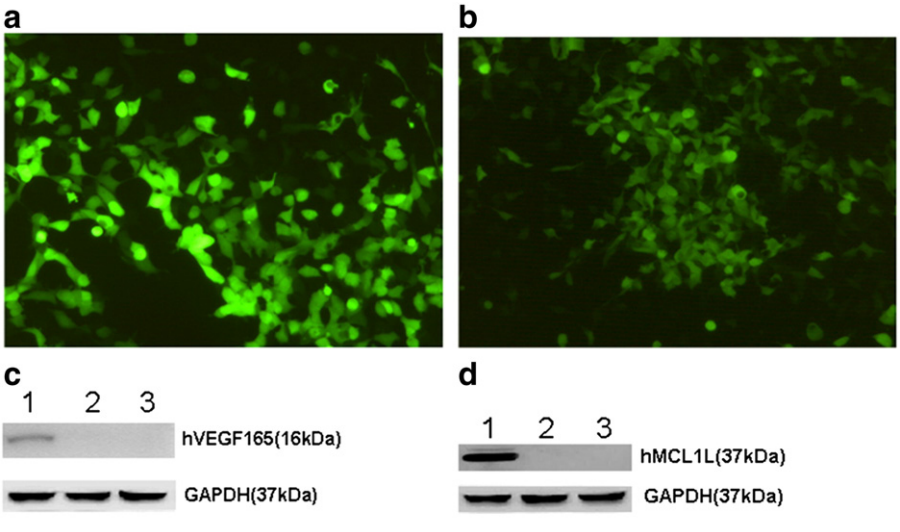

Fig. 3. Packaging and Western blot assay of adenovirus recombinants. (a) Adenovirus recombinant (hVEGF165) derived from pAdTrack-CMV expressing GFP was transfected into 293FT cells. Transfected cells were examined under a fluorescence microscope 8 d after transfection. Magnification, ×200. (b) Adenovirus recombinant (hMCL1) derived from pAdTrack-CMV expressing GFP was transfected into 293FT cells. Transfected cells were examined under a fluorescence microscope 8 d after transfection. Magnification, ×200. (c) Western blot assay for hVEGF165 protein expression in COS7 cells after recombinant adenovirus infection. (d) Western blot assay for hMCL1 protein expression in COS7 cells after recombinant adenovirus infection. Grouping: 1. COS7 cells infected with appropriate recombinant adenovirus; 2. COS7 cells infected with empty adenovirus; 3. COS7 cells without infection with adenovirus.

backbone plasmids, the amplified area of the primer extends an approximately 30 kb adenovirus backbone sequence. However, it is very difficult for commonly used Taq polymerase to efficiently amplify such a long sequence (Fig. 1). Moreover, considering the high sensitivity of PCR, negative controls were established in the process of colony PCR to evaluate PCR contamination, ensure the reliability of PCR results, and carefully manipulate the PCR system to maximally reduce the incidence of false positives. Also, positive controls were used to evaluate PCR efficiency, and to maximally reduce the incidence of false negatives. By doing this, once recombination of the shuttle and pAdEasy adenovirus backbone plasmids was completed in 16–20 h, we chose clones for colony PCR selection, which could determine the recombinants in a short time-frame (Fig. 2a). Clones 1, 3–6 and 8–9 were possible recombinants. In addition, a series of shuttle plasmids such as pShuttle-CMV and pSES-HUS, which could be used for over-expression or knock-down of target genes in the AdEasy system, has shown a similar shuttle plasmid sequence using the same strategy of recombination [9]. In our experiment, we successfully applied the negative selection method of colony PCR to perform recombinant selection and improve adenovirus packaging.

Second, while we applied colony PCR to identify recombinants, we cultured the colonies in LB medium. To maximally reduce the damage of RecA activity to the recombined backbone plasmid in BJ5183 cells, culturing time was limited to less than 10 h [7]. Because DNA concentration was too low, we did not perform PacI enzyme digestion for electrophoretic identification, but retransformed DNA into DH10B cells or other common strains used for plasmid propagation. The purified recombinant DNA from DH10B cells was used to perform PacI digestion (Fig. 2b), indicating that the negative selection method using colony PCR, and PacI enzyme identification achieved similar results.

Third, to maximally decrease the damaging side effect of RecA activity in BJ5183 cells to important elements of adenovirus recombination, and to increase the success rate of packaging and decrease the consumption of transfection reagent, we extracted and mixed several adenovirus recombinants ($n \geq 3$) in an equivalent mixture containing the same target gene, which were digested with PacI to liberate both ITRs. We then purified with ethanol precipitation and transfected into 293FT packaging cells for adenovirus packaging [4,10]. Eight days after transfection, the GFP marker gene showed a cytopathic effect, indicating successful adenovirus packaging (Fig. 3a, b). In addition, COS7 cells with zero-background expression of target genes were infected with culture supernatant containing adenovirus from 293FT cells, then specific over-expression of proteins was determined by immunoblotting (Fig. 3c, d).

In summary, the negative selection method to identify AdEasy adenovirus recombinants by colony PCR can identify the recombined colony within a short time-period, and maximally avoid damage to the recombinant plasmid by limiting recombination time, resulting in improved adenovirus packaging. In comparison with the traditional identification method of PacI digestion, this method is specific and economical, which may promote experimentation based on adenoviruses, and provide a reference for similar recombinant screening.

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