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Synthesis a New Viral Base Vector Carrying Single Guide RNA (sgRNA) and Green Florescent Protein (GFP)

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Abstract

CRISPR/Cas9 system is a powerful gene editing tool *in vivo* and *in vitro*. Currently, CRISPR/Cas9 delivery cells or tissue with different vehicles are available, and Adeno-associated virus (AAV) in one of them. Due to AAV packaging size limitation, AAV base vectors that carry CRISPR/Cas9 system do not have florescent tag like GFP for simple detection and navigation of cells, containing AAV. The aim of this study was to modify and synthesis AAV base vector for CRISPR/cas9 system containing sgRNA and GFP.Px602 plasmid was double digested with NcoI and HindIII restriction enzyme. *Gfp* gene was amplified from px458 plasmid. Linear digested px602 and amplified *Gfp* gene were ligated together. After transformation and colony PCR on white colonies, plasmid was extracted and transfected to HEK-293 cell line. *Gfp* expression was monitored by florescent microscopy. After transfection of modified plasmid, florescent microscopy of HEK-293 cells showed shining green florescent cells, which indicate that *Gfp* gene, was replaced in the correct place according to our design.We modified an AAV base vector carrying CRISPR/Cas9 system, and synthesized a new vector carrying *Gfp* gene and sgRNA that can be packaged as reporter AAV for navigation and detection of cells, containing AAV.

Keywords: CRISPR/Cas; AAV base vector; Gene editing.

Introduction

CRISPR/Cas system is an adaptive immune system in archaea and bacteria that prevents foreign genetic element invasion and phages to their genome [1-3]. Today CRISPR/Cas9 system has been engineered to be used as a powerful gene editing tools in vivo and in vitro [4-6]. There are three types of CRISPR/Cas systems, but type 2 (CRISPR/Cas9) is simpler than the others, which has been described in more details [7-11]. CRISPR/Cas9 system consist of a short non coding RNA (guide RNA or crRNA) and a trans activating RNA (tracRNA) (combination of both RNAs known as sgRNA) that combine with programmable endonuclease Cas9 and induce double strand break in its target sequence. This causes endogenous repair in 2 ways: 1.

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NHEJ (non-homologous end-joining, where repair occurs without template strand, so it is error prone, 2. HDR (homology directed repeat) needs exogenous sequence strand to repair, so it has high fidelity and specificity [12-15]. One of the vehicles for CRISPR/Cas9 system delivery to cells or tissues is Adeno associated virus (AAV), which is a deficient virus that infects human, but cannot cause disease [16-18]. Since AAV does not have pathogenicity, immunogenicity and tumorigenicity, it is an interesting vehicle for CRISPR/Cas9 delivery [19,20]. AAV has divers serotype that can target different tissues so it increases target specific delivery [21]. Although AAVs have several advantage to deliver CRISPR/Cas9 system for gene editing, its most important drawback is the small size of genome (4.7kb) and limitation in cargo size, so it cannot carry standard CRISPR/Cas9 (containing streptococcus pyogenes or sp Cas9). To solve this problem, researchers have described another type of Cas9, saCas9 (staphylococcus aureus**)** that is 1kb smaller than spCas9, but has the same efficiency for package in AAV [21,22]. Commercial AAV base vector that carries sgRNA and saCas9 is available but due to limitation in packaging, these vectors do not have simple florescent tag, such as GFP, mCherry and etc for navigation of virus. The aim of our study was to produce AAV base vector that can carry GFP tag and sgRNA construct.

Materials and Methods

Synthesis of plasmid: Plasmid PX602 was purchased from addgene. According to the map sequence of plasmid, the restriction enzymes HindIII (New England Biolab) and NcoI(New England Biolab) were selected and the plasmid was digested with them. After double digest, the linear plasmid digested with those enzymes, was extracted (QIAquick gel extraction kit) from 0.5% agarose gel after electrophoresis. Green florescent protein gene was amplified from px458 plasmid by primers that carried suitable linker sequence for the those restriction enzymes (forward primer had linker sequence for NcoI and Reverse primer had linker sequence for HindIII). PCR assey was done by the following program : 1 cycle (95 Cº, 2 Min) ; 30 cycle (95 Cº , 30s ; 56Cº , 30s; 72Cº , 30s) ; 1 (cycle 72 Cº, 5min) and 4Cº for ∞. The amplified products were separated by electrophoresis on a 1.5% (w/v) agarose gel and extracted using QIAquick gel extraction kit. To produce single strand overhang that can be fitted in the linear vector, Gfp gene was double digested with those enzymes. Then linear plasmid and Gfp gene were ligated together by T4 DNA ligase enzyme (New England Biolab).

Cloning of plasmid: Competent cells were produced from *Escherichia coli DH5α* according to the manufactures instruction. Both plasmids (PX602 and modified PX602) carry ampicillin resistance gene as reporter genes. Plasmid was cloned in competent cells using the fallowing method: 2 µl of the plasmids were added to 50µl of competent cells, and mixed, then placed on ice box for 30 min, 90s in 42Cº and 10 min on the ice box again respectively. After that 450µl LB broth was added to the mixture and shaken for 1hr in 37Cº. Then, the mixture was cultured in LB agar (Merck) that was supplemented with ampicillin and incubate for 18hrs in 37Cº. If the plasmid was uptaked by the competent cells, can induce ampicillin resistance and growth in LB agar plate which supplemented by ampicillin. After 18hrs, the presence of white colonies was an indication that the plasmid had entered the competent cells.

Colony PCR and plasmid extraction: Coloning PCR was performed to confirm that withe colonies carry modified plasmid containing Gfp gene in their sequence. After that modified plasmid was extracted (QIAprep spin miniprep kit) to confirm colonies, all according to the manufacturer instructions.

Cell culture and transfection and monitoring: Human embryonic kidney 293 were cultured in F12 DMEM (Dulbecco's Modified Eagle's Medium) media that was supplemented with 10% FBS (Fetal Bovine Serum) (Gibco) and 1% pen-strep (Penicillin-Streptomycin)(Thermo Fisher Scientific). After 2 subculture cells were transferred to the 6 well plate and seeded with Opti-mem media (*Reduced Serum Media* Thermo Fisher Scientific) after 24 hrs, modified vector were transfected to the cells (with 60% confluency) by lipofectamine 2000 (Invitrogen) according to the manufacturer instruction. After 8hrs of transfection, Opti-mem media were discarded from cells and full and fresh DMEM were added to the cells. After 48 to 72hrs, cells were monitored by florescent microscopy.

Results

Gfp gene was amplified, using the designed primers from plasmid px458. After gel electrophoresis a 714 bp fragment was extracted from gel. Linear plasmid px602 that was digested with both NcoI and HindIII was extracted from gel. After ligation of *Gfp* gene and linear vector and transformation to the *DH5α*, white colonies appeared in the LB agar plate supplemented with ampicillin. *DH5α* does not have any ampicillin resistance gene in its genetic map sequence, so naturally cannot grow in ampicilin supplemented media.

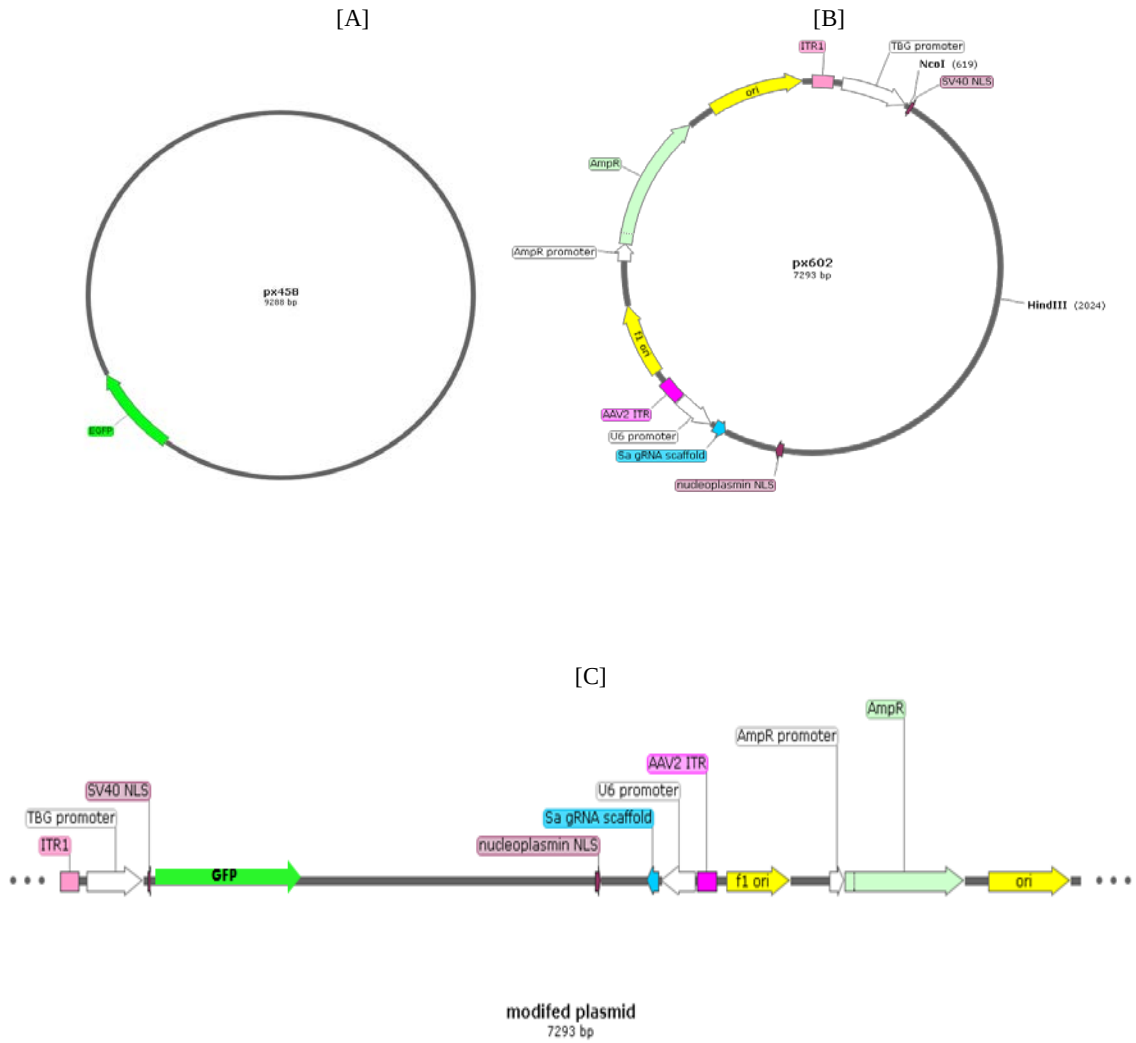

Figure 1. schematic plasmids map. [A] Px458 plasmid that *Gfp* gene amplified from it. [B] Px602 plasmid and cut site for NcoI and HindIII. [C] Linear form of modified plasmid

Consequently, appearance of white colonies shows that the circular vector with ampcillin gene was successfully transferred to them. To confirm and select white colonies that carried modified plasmid, colony PCR was performed and a 714bp fragment was seen again (Fig. 1). Plasmid was extracted with optimal density 420µg/µl. According to the manufacturer instruction, 24hrs after HEK-293 seeding in a 6 well plate, the modified plasmid was transfected to the cells by lipofectamine 2000. GFP production was seen after 24hrs and monitored by florescent microscopy (Fig. 2). Shining green florescent in the cells showed that (according to our design) *Gfp* gene was replaced in the correct place in px602 vector and successfully expressed in the cells.

Discussion

An attractive vehicle to deliver CRISPR/Cas system is AAVs, which have several advantages such as: 1, AAVs have different serotype that increase target specificity, 2) Naturally they are not immunogenic and 3) in contrast to the lentivectors, AAVs do not have the risk of oncogenicity [19-20]. Although AAVs have more advantages, they have an important disadvantage that limits there usage, such as cargo size restriction as the most important obstacle for packaging AAVs [17]. To overcome this limitation, researchers described a shorter Cas9 enzyme; saCas9, which is 1Kb smaller than spCas9, but has the same efficiency [22]. Nowadays, AAV vectors that carry saCas9 and one

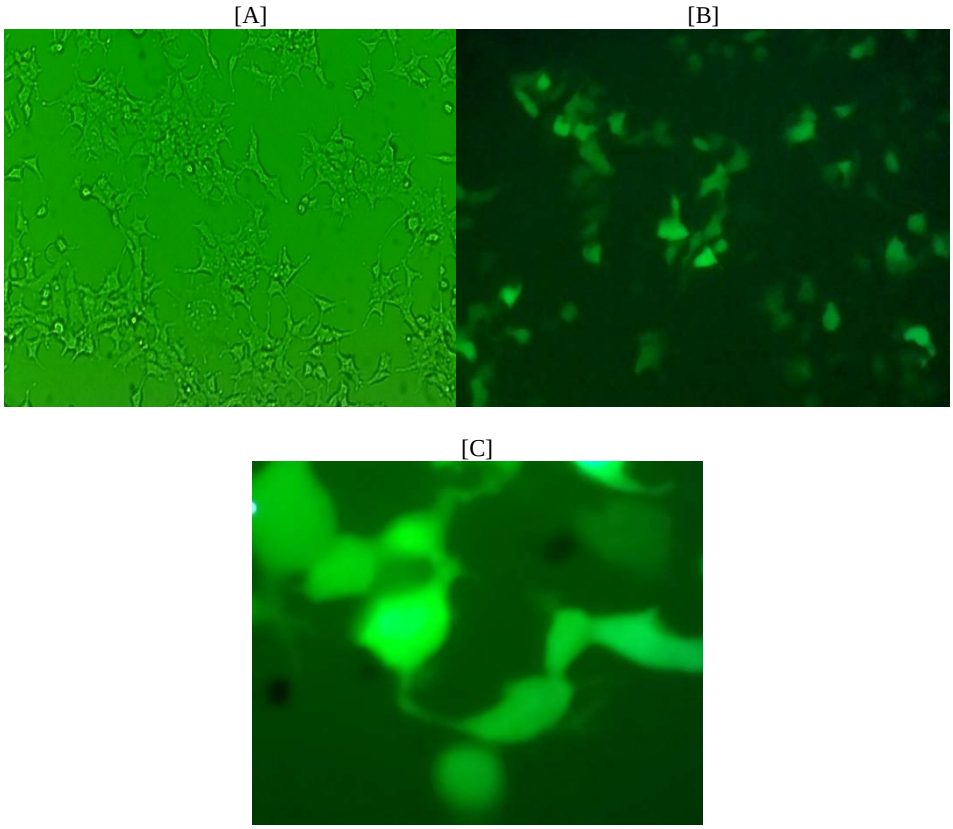

Figure 2. [A] HEK-293 cell by green filter of light microscopy. [B] Florescent microcopy of transfected (x20) cells. [C] Florescent microcopy of transfected (x40) cells.

sgRNA are commercially available. Because of AAVs cargo size limitation, these vectors do not have common florescent tag, such as GFP. However, use of Gibson assembly cloning method is an option for synthesis of the vector [24] that carry saCas9, sgRNA and GFP, but this construct is more than AAV capacity for packaging. Hence, for navigation of cells that contain AAV, we modified a commercial vector to make a new vector that carry *Gfp* gene and sgRNA and can be packaged as a reporter AAV. This AAV reporter is useful in both navigation of AAVs that enter the cell and in double viral infection system for gene editing *in vitro* and *in vivo*. According to the modified vector, in double viral system, one AAV carry saCas9 and sgRNA and the other AAV acts as a reporter that carries GFP and sgRNA. Although assembling all subunits is necessary for gene editing in one AAV construct, it is more efficient than double viral system. Furthermore, a simple reporter tag such as GFP is useful for detection and navigation and isolation of cells that contain AAV. Therefore, these cells have a chance of gene editing by CRISPR/Cas system. Due to AAV packing size limitation, routine AAV base vector that carry CRISPR/Cas system do not have florescent tag like GFP. However, GFP is useful for detection and navigation of cells that contain AAV. Hence, we modified and synthesized new AAV base vectors that carry both sgRNA and *Gfp* gene. We believe that this new vector is useful in double viral system for delivery of CRISPR/Cas9 system.

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