Optimization of a multiplex CRISPR/Cas system for use as an antiviral therapeutic


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Abstract

RNA-guided endonucleases or CRISPR/Cas systems have been widely employed for gene engineering/DNA editing applications, and have recently been used against a variety of dsDNA viruses as a potential therapeutic. However, in vivo delivery to specific tissue reservoirs using adeno-associated virus (AAV) vectors is problematic due to the large coding requirement for the principal effector commonly used in these applications, Streptococcus pyogenes (Spy) Cas9. Here we describe design of a minimal CRISPR/Cas system that is capable of multiplexing and can be packaged into a single AAV vector. This system consists of the small Type II Cas9 protein from Staphylococcus aureus (Sau) driven by a truncated CMV promoter/enhancer, and flanked 3' by a poly(A) addition signal, as well as two sgRNA expression cassettes driven by either U6 or ~70-bp tRNA-derived Pol III promoters. Specific protocols for construction of these AAV vector scaffolds, shuttle cloning of their contents into AAV and lentiviral backbones, and a quantitative luciferase assay capable of screening for optimal sgRNAs, are detailed. These protocols can facilitate construction of AAV vectors that have optimal multiplexed sgRNA expression and function. These will have potential utility in multiplex applications, including in antiviral therapy in tissues chronically infected with a pathogenic DNA virus.

Keywords: AAV multiplex genome editing CRISPR/Cas9 Staphylococcus aureus Cas9 tRNA promoters Quantitative sgRNA screening method Viral therapeutic

1. Introduction

RNA-guided dsDNA endonucleases from bacteria have the potential to revolutionize broad areas of molecular biology. The bulk of this work has focused upon genome engineering/editing, such as gene knockouts or knockins in cultured cells or in vivo. Therapeutic applications have been proposed as well, and these are generally focused on the gene therapy of congenital genetic diseases [1]. Distinct from the correction of inherited disease, other therapies could exploit the specific disruption of genes associated with chronic viral disease. Numerous reports have recently employed Streptococcus pyogenes (Spy) Cas9/sgRNA combinations to disrupt or eliminate pathogenic viruses including human immunodeficiency virus 1 (HIV-1), human papilloma virus (HPV), hepatitis B virus (HBV) or Epstein-Barr virus (EBV) in infected cells [2–8].

In the case of chronic or aberrant viral infection resulting in malignancy, as seen in the case of HBV and HPV respectively, disruption of viral genes represents a potentially highly effective means of eliminating the virus or infected, transformed host cells [4–7]. Specifically, in the case of HBV, a dsDNA viral intermediate, the highly stable, viral covalently closed circular DNA (cccDNA), enables persistence and replication in the infected liver [9]. Similarly, in the case of HPV, integrated viral DNA genomes expressing the HPV E6 and E7 oncogenes drive the transformation of cervical, anal, and throat epithelia, resulting in malignancies in those tissues [10]. Our group, among others, has shown that CRISPR/Cas systems can be highly effective means of eliminating HBV from infected cells and, in the case of HPV-transformed cells, targeting E6 and E7 efficiently induces specific tumor cell killing [5–7,11].

Despite these early proof of concept studies, the efficient delivery of CRISPR/Cas systems to infected cells in vivo remains problematic. AAV represents an ideal viral vector for transduction of liver and tumor tissue and has been employed widely to deliver various payloads to similar tissues. However, the packaging size of AAV vectors, at ~4.5 kb excluding the essential inverted terminal repeats (ITRs), is limiting, and the commonly used Spy Cas9 gene, at ~4.2 kb, is clearly too large to permit the construction of recombinant AAV vectors expressing both Cas9 and an sgRNA [12]. In
contrast, the recently described *Staphylococcus aureus* (Sau) Cas9, which is encoded by an ∼3.2-kb gene, appears ideal for this application, and we have independently confirmed details of its characterization [13 and data not shown]. In this work, we describe an AAV vector platform capable of multiplex sgRNA expression utilizing previously described tRNA promoters [14,15]. Also, we have included a facile method of inserting the identical multiplex sgRNA/Cas9 cassette into a lentiviral platform, which may be more ideal in some contexts such as lymphocyte transduction. More importantly, we demonstrate how to clone and then screen sgRNA panels for highly active candidates that would be optimal for therapeutic applications, which can be incorporated into the final AAV and lentiviral vectors for packaging.

2. Materials

- CaPO₄ prepared as described in [16].
  - 2.5 M CaCl₂: 183.7 g CaCl₂·2H₂O, H₂O to 500 ml. Store at −20 °C in 10-ml aliquots.
  - 2× HBS solution: 16.4 g NaCl (0.28 M), 11.9 g HEPES, 0.21 g Na₂HPO₄ (1.5 mM), H₂O to 800 ml. Titrate to pH 7.05 with 5 N NaOH. Bring final volume to 1 liter with H₂O. Store at −20 °C in 10 ml aliquots.
- Dual-Luciferase Reporter Assay System (E1910; Promega).
- SURE 2 cells (200152; Agilent).
- Terrific Broth (Invitrogen, 22711-022).

3. sgRNA cloning and screening for optimal activity

Previous experience with TALENs and Spy Cas9 has taught us that there is a wide range of efficacy for both effectors, as assessed by end-point reporter assays. Assays that rely on duplex cleavage, such as Surveyor (Transgenomic), are useful but only semi-quantitative and lacking in sensitivity. To improve sensitivity, we developed a novel quantitative assay. We inserted an HIV-1 Rev-derived epitope tag at the amino-terminus of an in-frame target, and this was then fused in-frame to a 3’ reporter gene (Fig. 1A). This platform is highly specific: when cells are co-transfected with a Cas9 effector and an eGFP reporter, we observe a marked reduction in eGFP-positive cells when the effector is specific for the indicator, and the few remaining eGFP-positive cells show greatly reduced mean fluorescence intensity (Fig. 1B and C). We have also confirmed by Western blot that these cells indeed have a greatly reduced level of the fusion protein subsequent to specific effector cleavage, as shown in a cross-talk experiment (Fig. 1D). In a further refinement, we have inserted firefly luciferase (FLuc) in place of eGFP, and this vector can be used in a dual-luciferase assay that is highly quantitative. To our surprise, when we screened numerous Sau Cas9 sgRNAs with this assay there was a great deal of variability in the end-point efficiency from candidate to candidate (Fig. 1E). These data confirm that where highly effective disruption of genes is required, screening multiple sgRNAs for a highly effective candidate is crucial.

3.1. Cloning putative sgRNAs into the final scaffold for initial screening

1. We generated a construct containing three expression cassettes for a human codon-optimized Sau Cas9 and two chimeric Sau sgRNAs driven either from a tRNA or U6 Pol III promoter – pSauCas9Dual (Fig. 2A).
2. Digest 1 μg of pSauCas9Dual using BsmBI to insert a target sequence into the sgRNA-1 expression cassette (Fig. 2A). Do not dephosphorylate the vector, and gel purify the linearized fragment of ∼7.5 kb.

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**Fig. 1.** Quantitative optimization of sgRNA cleavage and selection by fusion protein assay. To quantitatively test the efficiency of engineered sgRNAs, we embedded virally derived targets in-frame between an HIV-1 Rev-derived epitope tag and a reporter gene (eGFP or FLuc). Specific cleavage at the target site will result in error prone repair via non-homologous end joining (NHEJ) leading to diminished expression of the Rev-indicator fusion protein. (A) Schematic representation of the Cas9/sgRNA indicator construct. A herpes simplex virus (HSV) or an HBV target containing a putative Spy PAM was cloned into the Rev-eGFP indicator variant. (B) % GFP positive is shown from the assay performed in (A). (C) GFP mean fluorescence intensity (MFI) is shown from the assay performed in (B). (D) Western blot analysis using a Rev-specific antiserum is shown for a panel of Rev-eGFP fusion proteins in the presence and absence of a specific sgRNA. (E) A Rev-luciferase based indicator assay was conducted on a set of Sau sgRNAs and their cognate reporters for U6 Sau Cas9 sgRNAs (N = 3 biological replicates; error bars represent SD).
3. Anneal oligonucleotides containing sgRNA target sequence 1 using 1× annealing buffer.
  1 µl (100 µM) of sgRNA1-F.
  1 µl (100 µM) of sgRNA1-R.
  48 µl (50 mM NaCl, 10 mM Tris pH 7.5) 1× annealing buffer.
  Total: 50 µl.
  Anneal in a thermocycler using the following parameters:
  95 °C for 5 min and then ramp down to 10 °C at 1 °C/20 s.
4. Dilute annealed oligonucleotides 1:100 in 1× annealing buffer.
  Ligate 2 µl of insert with 2 µl BsmBI digested psauCas9Dual in a
  20-µl reaction.
5. Mix the annealed target + PAM oligonucleotides to generate
  the in-frame Rev-ORF fusion protein, computationally translate the
  sgRNA target sequence into amino acids.
6. To quantitatively test the efficiency of sgRNA-1 and sgRNA-2,
  design oligonucleotide pairs containing the target sequences,
  along with a proper Sau Cas9 PAM (5′-NNGRRT-3′), to be
  embedded in-frame between the HIV-1 Rev epitope tag and the indicator gene
  (Fig. 2B). To verify the in-frame Rev-indicator-ORF fusion protein, computationally translate the
  sgRNA target sequence into amino acids.
7. Digest 1 µg of pcDNA-Rev-Luc with BamHI and EcoRI and clone
  the annealed target + PAM oligonucleotides to generate
  sgRNA-1 and sgRNA-2-containing GFP or Fluc-based indicator
  constructs (Fig. 2B). Do not dephosphorylate the digested backbone, and repeat the procedure described above.

3.2. Screening putative sgRNAs by dual luciferase assay to obtain
  highly efficient sgRNAs (see Fig. 2C)

1. 293T cells are maintained in Dulbecco’s modified Eagle medium
  (DMEM) +10% fetal bovine serum (FBS), 2 mM antibiotic-
  antimycotic (Gibco Cell Culture), and 50 µg/ml gentamicin
  (Life Technologies) at 37 °C.
2. 293T cells were plated at 1.25 × 10^5 cells per well in 12-well
  plates and transfected using the calcium phosphate method
  with a 4:1 ratio of the sgRNA expression vector to the indicator
  plasmid. Transfection cocktails per well are mixed as follows:
  psauCas9Dual (effector): 1 µg.
  pcDNA-Rev-FLuc reporter: 250 ng (firefly luciferase).
  pCMV-RLuc internal control: 50 ng (Renilla luciferase).
  Add H2O to 45 µl total volume.
  +5 µl 10× calcium phosphate (first).
  +50 µl 2× HBS (second).
  Briefly vortex and incubate for 10 min at room temperature.
  Add transfection cocktail dropwise onto 293T cells.

3. 24 h post transfection, change media.
4. Assay for luciferase activity 72 h post-transfection using a Dual
  Luciferase Kit (Promega) or by flow cytometry (eGFP).

4. Optimization of the vector scaffold and shuttle cloning into
  lentiviral and AAV vectors

We have engineered a minimal scaffold below the AAV
  packaging limit of ~4.5 kb as depicted in Fig. 3A. First, we codon
  optimized and synthesized the Sau Cas9 gene [15]. Next, we opti-
  mized sgRNA and Cas9 expression cassettes for size and function.
  Previously, we have characterized ~70-bp human tRNA-derived
  Pol III promoters that are substantially smaller than the commonly
  used, ~250-bp U6 Pol III promoter yet highly effective for sgRNA
  expression [15]. If desired, these are compatible with the U6
  promoter for dual sgRNA expression, as shown in Fig. 3A and are
  highly functional as determined by a Rev-Fluc indicator assay
  (Fig. 3B). High Cas9 expression is also required for optimal func-
  tion, and we have compared a number of smaller Pol II promoters,
  as shown in Fig. 3C. A number of truncated variants of the human
cytomegalovirus (HCMV) immediate early promoter/enhancer are sufficient for high expression when compared to the full HCMV promoter/enhancer (left lane Fig. 3C). For gene editing applications in non-dividing cells, nuclear localization is essential. We confirmed that our Sau Cas9 was localized to the nucleus (Fig. 3D). In addition, no significant toxicity was observed with increased Sau Cas9 expression. The Sau sgRNA/Cas9 scaffold, which we have shown to be highly functional, can be shuttle cloned into either an AAV or lentiviral vector for vector-mediated delivery of genome editing components into target tissues in vivo or cells in culture.

4.1. Shuttle cloning the scaffold into the destination AAV vector

1. To insert the dual Sau Cas9 sgRNA scaffold into an AAV vector, digest 2 µg pTR-UF11 [17] KpnI and SphI for at least 1 h and add 1 µl of calf alkaline intestinal phosphatase (NEB) for 20 min. This plasmid contains two AAV2 ITR flanking sequences. Sau sgRNA/Cas9 expressions cassettes can be placed between these ITRs to make a dual sgRNA AAV vector (Fig. 4). Isolate the ~3.2 kb DNA vector fragment on a 0.7% agarose gel (the lower of two bands).

2. Digest 10 µg of pSauCas9Dual with KpnI and SphI to generate the Sau Cas9 and dual sgRNA expression insert. Isolate the ~4.3 kb DNA fragment on a 0.7% agarose gel.

3. Ligate purified vector and insert DNA in a 10 µl ligation reaction for at least 30 min at room temperature.

4. Chemically transform the entire ligation reaction into 100 µl of E. coli competent cells (Stratagene; 200152) and plate on an LB/Amp agar plate. Incubate for no more than 15 h at 30 °C to avoid recombination and loss of the ITRs.

5. Inoculate individual colonies into Terrific Broth (TB) (Invitrogen, 22711-022) medium to screen for correct clones. Grow TB cultures for no more than 15 h at 30 °C.

6. Purify DNA from bacterial cultures using a Plasmid MiniPrep kit (Thermo Scientific; K0503).

7. To identify positive SauCas9/sgRNA AAV vectors, digest mini-prep DNAs with BamHI and Smal. Clones with a correct SauCas9/sgRNA insert and intact ITRs will generate a ~620 bp, ~3.8 kb, and ~3 kb DNA fragment signature. These can then be used to carefully inoculate an overnight 250 ml culture for 15 h at 30 °C again to avoid ITR loss. These final preparations should then be validated by digest.

8. Select a commercial packaging facility, for example, the University of North Carolina Vector Core (Chapel Hill, NC), to package the final AAV vector on a fee for service basis, or invest effort in-house to develop the packaging expertise [18–20].
4.2. Shuttle cloning the scaffold into the destination lentiviral vector

We developed a destination lentiviral transfer vector, pLSauShuttle, which is amenable to shuttling the dual sgRNA/Sau Cas9 expression cassette from pSauCas9Dual. To insert the dual Sau Cas9 sgRNA scaffold into the lentiviral transfer vector, digest 2 μg of pLSauShuttle with BsmBI for at least 1 h, add 1 μl of calf alkaline intestinal phosphatase (NEB) for 20 min and gel purify the linearized fragment (Fig. 4).

2. Digest 2 μg of pSauCas9Dual with Asp718I (Sigma) and Sal I to generate the Sau Cas9 and dual sgRNA expression insert. Isolate the ~4.2 kb DNA fragment on a 0.7% agarose gel.

3. Ligate purified vector and insert DNA in a 10 μl ligation reaction for at least 30 min at room temperature.

4. Chemically transform ligations using DH5-alpha competent cells. Screen potential clones by restriction enzymes BamHI and EcoRI. Lentiviral clones containing the proper Sau Cas9 dual sgRNA insert will generate ~5.9 kb, ~3.5 kb, and ~550 bp DNA fragments.

5. Lentiviral vector particles are produced by co-transfection of plasmids coding for the virion packaging system (pCMV-R8.2, Addgene #12263), the transfer vector carrying the gene of interest (pLSauShuttle), and the VSV-glycoprotein (pMD2; Addgene #12259). 293T cells are plated in 15 cm² dishes at 50–60% confluency and transfected with 17 μg of pCMV-R8.2, 17 μg of pLSauShuttle, and 7 μg of pMD2g using the polyethyleneimine (PEI) method [21].

5. Conclusion

Here we have described the design and optimization of a multiplex capable AAV vector scaffold, cloning and screening of optimal sgRNAs for therapeutic applications, and how to assemble the final delivery vector. Use of AAV can greatly facilitate the delivery of CRISPR/Cas to liver, neuronal, muscle, and tumor tissues in vivo.

Dual sgRNA expression greatly extends the therapeutic potential of CRISPR/Cas9 systems. In the context of targeting viral DNA, dual sgRNA expression facilitates dual mutagenesis in series, and in parallel, structural disruption and/or large deletions. Multiplex targeting is also crucial to eliminating functional in-frame mutations that one could imagine being selected in an ensemble of mutants generated by single sgRNAs in vivo. Furthermore, a dual sgRNA vector could be readily adapted for staggered nickase use by addition of the Cas9 nickase recently described for Sau Cas9 [13,22] and this can greatly enhance cleavage specificity and thereby eliminate off target DNA cleavage. Delivery of multiplex sgRNA/Cas9 AAV vectors has the potential to greatly extend the use of the CRISPR/Cas system in vivo.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ymeth.2015.08.012.

References