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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.

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

Abbreviations: AAV, adeno-associated virus; CRISPR, clustered regularly interspaced short palindromic repeats; Sau Cas9, *Staphylococcus aureus* Cas9; sgRNA, single guide RNA; ITRs, inverted terminal repeats; PAM, protospacer adjacent motif.

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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

- $CaPO_4$ prepared as described in [16].
  - 2.5 M $CaCl_2$:183.7 g $CaCl_2{\cdot}2H_2O$, H_2O 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 Na_2HPO_4 (1.5 mM), H_2O to 800 ml. Titrate to pH 7.05 with 5 N NaOH. Bring final volume to 1 liter with H_2O. 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.

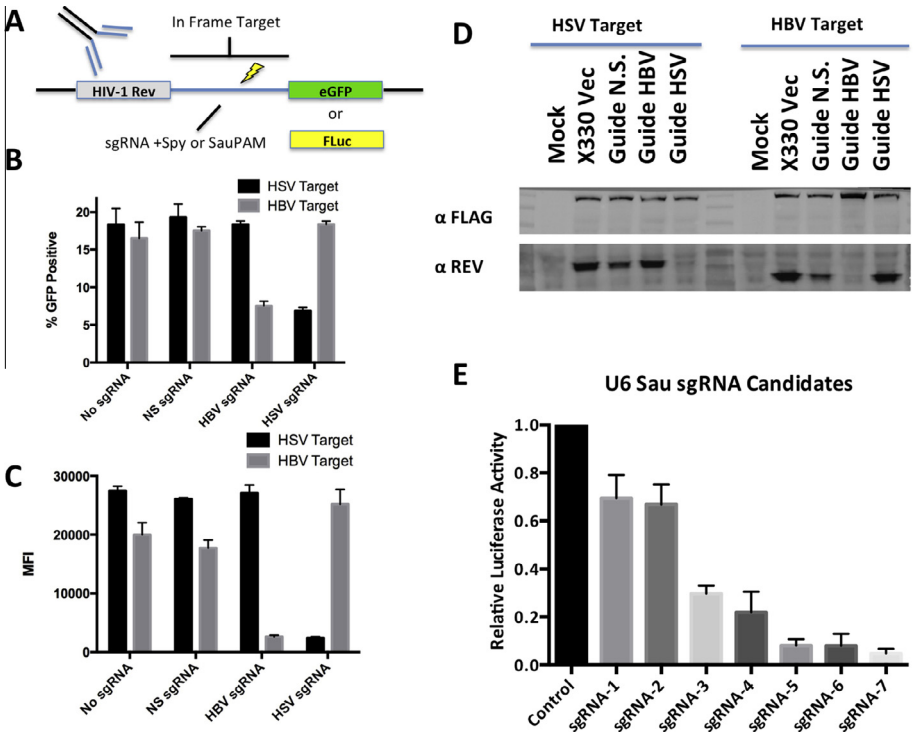

Fig. 1. Quantitative optimization of sgRNA cleavage and selection by fusion protein assay. To quantitatively test the efficiency of engineered sgRNAs, we embedded virally derived targets in-frame between an HIV-1 Rev-derived epitope tag and a reporter gene (eGFP or FLuc). Specific cleavage at the target 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 are shown for cells cotransfected as indicated. (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. Following successful cloning of sgRNA-1 into pSauCas9Dual, digest the dual sgRNA construct now harboring sgRNA target sequence 1 with BbsI and insert sgRNA target sequence 2 following the same procedure as previously described (Fig. 2A).
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 of interest to identify the presence of stop codons. This is typically only necessary for non-coding targets. If a stop codon is present, shift the frame of the target sequence until a contiguous coding segment is obtained.
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 detailed 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 H_2O 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 optimized 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 function, and we have compared a number of smaller Pol II promoters, as shown in Fig. 3C. A number of truncated variants of the human

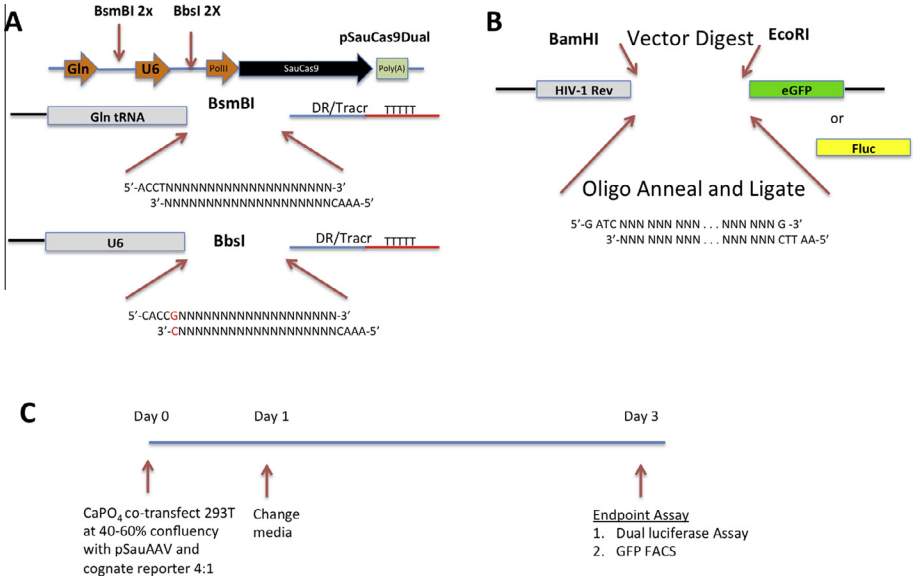

Fig. 2. Cloning schematic for sgRNA cloning into tRNA dual scaffold and reporter construct. Target selection, dual Sau Cas9/sgRNA and reporter reagent preparation, and reporter assay procedure are depicted. For *in vivo* editing using the Sau Cas9 system, 21–24 nt targets must be followed by a 5′-NNGRRT-3′ PAM at their 3′ end, where R designates a purine and *N* any nucleotide. The PAM should be included 3′ of the target for the Cas9 in question. (A) Schematic for cloning sgRNA oligonucleotides into either the glutamine tRNA or U6-based expression cassette. Digestion of pSauCas9Dual with either BsmBI or BbsI allows the directional insertion of annealed sgRNA oligonucleotide pairs into the Gln or U6 expression cassettes. A G-C base pair at the 5′ end of the Sau sgRNA is required for U6, but not tRNA-mediated transcription initiation. Note the differing nucleotide overhangs required for ligation into pSauCas9Dual. (B) For generation of a Rev-eGFP or Rev-FLuc reporter to test efficiency of Sau sgRNAs expressed from pAAVSauCas9, annealed oligonucleotides containing a virally derived DNA target, flanked 3′ by the Sau PAM, can be ligated in-frame into the reporter construct following digestion with BamHI and EcoRI. (C) Sau sgRNA reporter experiment schematic. 293T cells are transfected using the calcium phosphate method with the sgRNA-effector and a specific reporter plasmid and assayed 3 days post transfection for eGFP or FLuc knockdown.

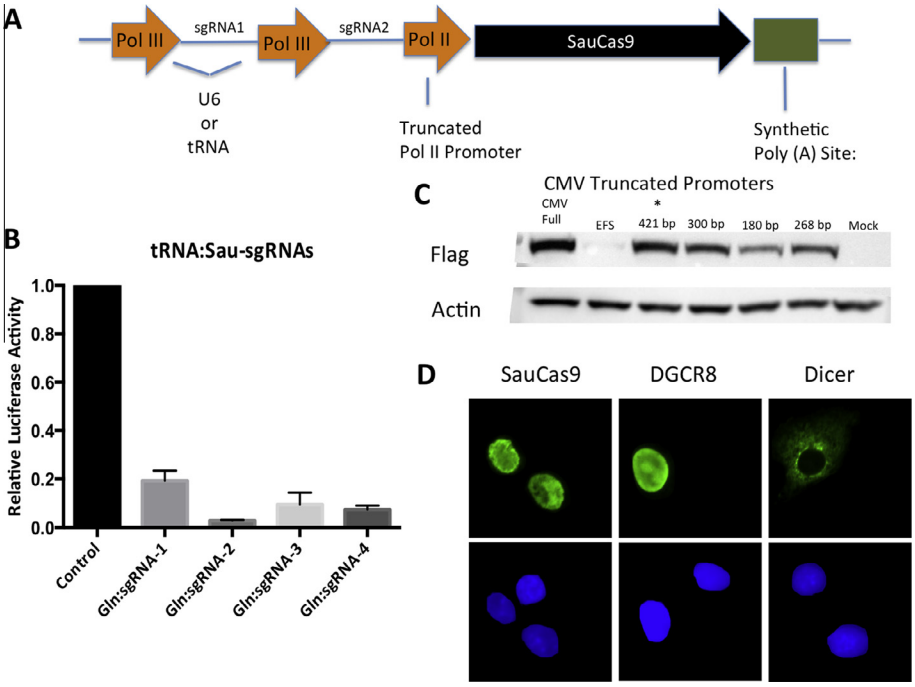

Fig. 3. Optimization of Sau Cas9 and sgRNA expression for use in a minimal vector cassette. Pol III and Pol II promoter adjustability for specific genome editing *in vivo*. (A) Schematic representation of a dual Pol III sgRNA and Pol II Sau Cas9 expression vector. sgRNAs can be expressed from either a U6 promoter or tRNA promoter in combination with a minimal CMV specific promoter for optimal target cleavage. (B) Sau Cas9 sgRNAs transcribed from a human glutamine (Gln) tRNA promoter were tested in a dual luciferase assay for function against a cognate reporter as described in Fig 1. ($N = 3$ biological replicates; error bars represent SD). (C) Western blot analysis is shown for Sau Cas9 expressed from CMV promoter truncations and the (EFS) promoter. We have selected a 398 bp derivative marked by an *, for further use. The sequences for pSauCas9Dual and pLSauShuttle are listed in Supplemental Fig. 1. (D) Immunofluorescence analysis is shown for Sau Cas9 (HA staining) expressed from the scaffold described in (A). DGCR8 and Dicer are nuclear and cytoplasmic controls respectively.

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 SURE2 competent *Escherichia coli* 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.

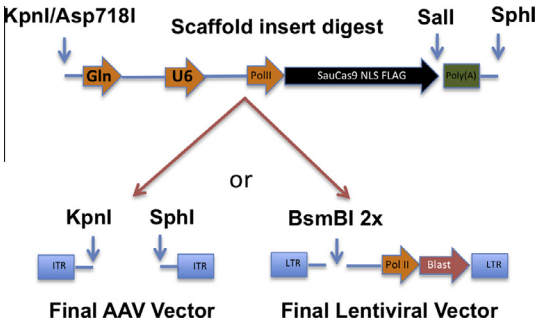

Fig. 4. Cloning schematic for shuttle cloning into lentiviral or AAV vectors. Shuttle cloning strategy for preparation of Sau Cas9/sgRNA expressing AAV and lentiviral viral vectors. To insert the Sau Cas9 dual sgRNA expression cassette into an AAV vector for *in vivo* multiplexed genome editing, digest pSauCas9Dual and an ITR containing AAV vector, pTR-UF11 [17], with KpnI and SphI. Similarly, digest pLSauShuttle and pSauCas9Dual with Asp718I/SalI, respectively, to generate a dual Sau Cas9 genome editing capable lentiviral transfer vector.

6. Purify DNA from bacterial cultures using a Plasmid MiniPrep kit (Thermo Scientific; K0503).
7. To identify positive SauCas9/sgRNA AAV vectors, digest miniprep DNAs with BamHI and SmaI. 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

1. We developed a destination lentiviral transfer vector, pLSauShuttle, which is amenable to shuttle cloning 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 using the 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 cm^2 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.

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