CRISPR/Cas9 nuclease cleavage combined with Gibson assembly for seamless cloning

Jia-Wang Wang[1], Amy Wang[1], Kunyu Li[1], Bangmei Wang[1], Shunqian Jin[2], Michelle Reiser[1], Richard F Lockey[1,3]

[1]Division of Allergy and Immunology, Department of Internal Medicine, University of South Florida Morsani College of Medicine, Tampa, FL, [2]Department of Pediatrics, Children's Hospital of Pittsburgh of University of Pittsburgh Medical Center and the University of Pittsburgh School of Medicine, Pittsburgh PA, and [3]James A. Haley Veterans' Hospital, Tampa, FL.

BioTechniques 58:161-170 (April 2015) doi 10.2144/000114261
Keywords: CRISPR cleavage; Gibson cloning; large vector; Cas9; single guide RNA

Supplementary material for this article is available at www.BioTechniques.com/article/114261.

Restriction enzymes have two major limitations for cloning: they cannot cleave at any desired location in a DNA sequence and may not cleave uniquely within a DNA sequence. In contrast, the clustered regularly interspaced short palindromic repeat (CRISPR)–associated enzyme 9 (Cas9), when coupled with single guide RNAs (sgRNA), has been used in vivo to cleave the genomes of many species at a single site, enabling generation of mutated cell lines and animals. The Cas9/sgRNA complex recognizes a 17–20 base target site, which can be of any sequence as long as it is located 5′ of the protospacer adjacent motif (PAM; sequence 5′-NRG, where R = G or A). Thus, it can be programmed to cleave almost anywhere with a stringency higher than that of one cleavage in a sequence of human genome size. Here, the Cas9 enzyme and a specific sgRNA were used to linearize a 22 kb plasmid in vitro. A DNA fragment was then inserted into the linearized vector seamlessly through Gibson assembly. Our technique can be used to directly, and seamlessly, clone fragments into vectors of any size as well as to modify existing constructs where no other methods are available.

Cloning is an essential tool for genetic engineering, with many cloning techniques having been developed over the years (1–8). Most of these approaches rely on cleaving DNA using restriction enzymes. Commonly used restriction enzymes have 6- or 8-bp recognition sites, which occur with a predicted frequency of 1 in every 4096 or 65,536 bp, respectively, in random sequence. When used in cloning, restriction enzymes have two limitations: (*i*) Since their cleavage sites are restricted to those determined by their recognition sequences, restriction enzymes cannot cleave at any arbitrary location that an investigator might wish, such as required for seamless cloning; and (*ii*) multiple cleavage sites often exist, especially in a large vector, which is a drawback in most cases since unique sites are often required for cloning.

To overcome these limitations, researchers have devised alternative methods, such as Gateway cloning (9), sequence and ligation-independent cloning (SLIC) (1), quick and clean cloning (QC) (2), and Gibson assembly (5), that eliminate the need for restriction enzyme digestion. These methods require a vector and a linear insert with sequences at each end that are homologous to one another. Gateway cloning requires the use of specific vectors, but does not depend on a linearized vector, whereas SLIC, QC, and Gibson assembly are not limited to specific vectors, but require enzyme digestion or inverse PCR to linearize the cloning vector. However, the use of inverse PCR to linearize the cloning vector is limited by the difficulty in amplifying vectors with high GC content, repeats, or long sequences. In addition, mutations can be introduced during the amplification of large vectors.

METHOD SUMMARY

A T7 transcription template for generating a single guide RNA (sgRNA) targeting a desired site in a cloning vector was synthesized by PCR from the sgRNA expression plasmid pX330. The sgRNA synthesized from the template by in vitro transcription was then used with the Cas9 enzyme to linearize a 22 kb vector in vitro, and a DNA fragment was seamlessly inserted into the cleavage site using Gibson assembly.

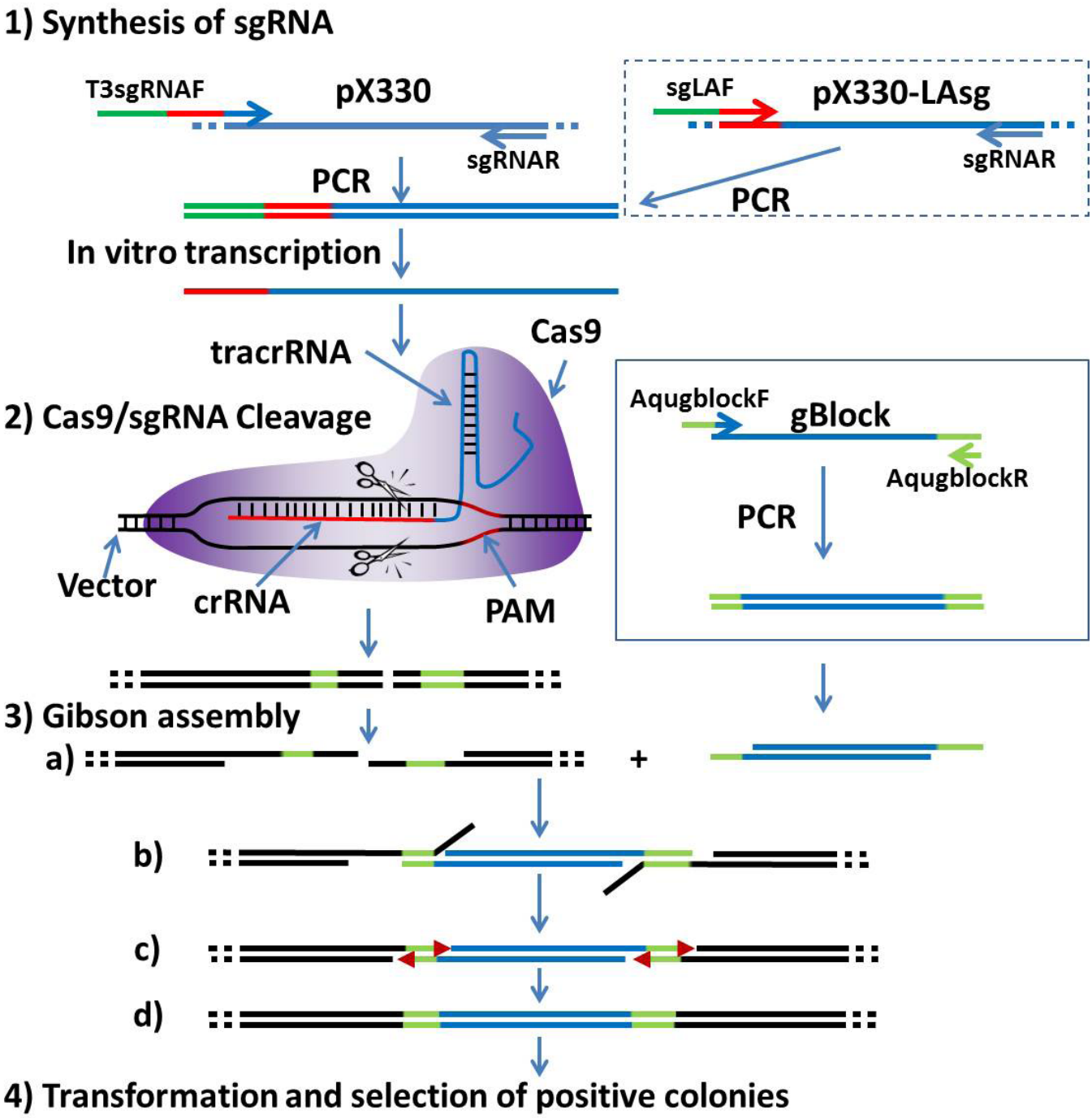

Figure 1. Schematic diagram of CRISPR/Gibson cloning method. 1) Synthesis of sgRNA: The duplex DNA template for in vitro transcription using T7 RNA polymerase was PCR synthesized from the pX330 vector. The T3gRNAF forward primer has three parts: a T7 promoter (green), a 19-base guide sequence for the T3 promoter (red), and the first 20 bases (blue) of the tracrRNA from pX330. A duplex DNA transcription template was also PCR synthesized from the pX330-gRNA vector (pX330-LAsg), as shown in the dashed rectangle. The PCR products were purified and transcribed in vitro with T7 RNA polymerase to synthesize the sgRNA (81 bases). 2) Cas9/sgRNA Cleavage: The purified sgRNA and Cas9 nuclease were mixed together to form the Cas9/sgRNA complex. The 19-base guide sequence then guided the complex to the targeted sequence to linearize the cloning vector. 3) Gibson assembly: The insert was PCR amplified (as shown in the solid rectangle) from a gBlock using a pair of primers so that it had 21 bp and 40 bp sequences at the left and right ends that overlapped with the vector sequences flanking the cleavage site. The homologous sequences at the ends of the insert and in the vector are shown in light green. The linearized cloning vector was purified and ligated with the insert in vitro using Gibson assembly. The ends of the linearized vector and inserts were chewed back using T5 exonuclease to produce 3′ overhangs that exposed the homologous sequences in the vector and insert (a) and were then annealed together (b). The protruding 3′ overhangs were removed by the 3′ to 5′ exonuclease activity of the DNA polymerase in the Gibson assembly mix (c). Repair with DNA polymerase and Taq ligase filled in the gaps and sealed the nicks to form a covalently closed double-stranded plasmid molecule (d). The red arrows indicate the filling in of gaps by the DNA polymerase. 4) Transformation and selection of positive colonies.

Direct and seamless cloning is impossible with these cloning methods when large vectors (cosmids, baculoviral or adenoviral vectors, or bacterial artificial chromosome (BAC) plasmids) are utilized. For example, to clone a fragment into a baculoviral or adenoviral vector, a smaller shuttle vector is typically used to clone the fragment, and then the cloned insert is transferred into a larger vector through homologous recombination in cells (10). This process is time-consuming, often taking a month or more to obtain the correct clone. Alternatively, these large vectors can be engineered to contain specific recombination sequences such as the attR1 and attR2 sites used for Gateway cloning (11). Although the Gateway-based method takes less than 2 weeks to obtain correct clones, the major disadvantage of such methods is that they can only be applied to specific vectors. Moreover, it is difficult, if not impossible, to modify an existing construct using these techniques, which can be desirable as well as cost-effective in certain studies.

To overcome these cloning limitations, we examined the use of the clustered regularly interspaced short palindromic repeat (CRISPR)–associated enzyme 9 (Cas9) in combination with the Gibson assembly technique to clone a DNA fragment seamlessly at virtually any user-specified location in a large vector. CRISPR is an adaptive immune system in bacteria that destroys naturally occurring and engineered phages and plasmids (12). It consists of: (*i*) CRISPR-associated protein 9 (Cas9), an endonuclease that cleaves a double-stranded DNA (dsDNA) target site; (*ii*) the CRISPR-related sequence (crRNA) containing the guide sequence that specifies the target sequence; and (*iii*) a trans-activating CRISPR-related RNA (tracrRNA) sequence from the bacterial CRISPR system that is hybridized to the crRNA. The 20-nucleotide guide sequence in the crRNA directs Cas9 to the complementary target site located immediately 5′ of the protospacer adjacent motif (PAM; sequence 5′-NRG, where R = G or A) that is required for guided DNA recognition and cleavage by the Cas9/crRNA/tracRNA complex (13,14). This system has been simplified for use as a genome editing tool by fusing the crRNA and tracRNA into one molecule, the single guide RNA (sgRNA). The modified CRISPR/Cas9 system has been successfully used to edit the genomes of many species (15–25). Here we demonstrate the feasibility and efficiency of cloning through the in vitro use of the CRISPR system to cleave a large vector at any desired location.

Materials and methods

Reagents and oligonucleotide synthesis: All enzymes and other reagents were purchased from New England Biolabs (NEB; Ipswich, MA) unless otherwise specified. DNA oligonucleotides were synthesized by Integrated DNA Technologies (IDT, Coralville, Iowa) or Eurofins Genomics (Huntsville, Alabama).

The protocol for seamless cloning using in vitro CRISPR/Cas9 nuclease cleavage combined with the Gibson assembly comprises the following four steps (Figure 1):

1. Synthesis of sgRNAs

a) sgRNA cloning: The guide sequence for the LA sequence was designed using the online CRISPR Design Tool (http://crispr.mit.edu/). Two oligonucleotides (mLAsgF and mLAsgR) were synthesized and annealed to form a double-stranded fragment with the desired overhangs (sequences in lower case in Table 1) for cloning into the pX330 vector (Addgene plasmid 42230) (16). The 2 oligonucleotides were diluted and mixed together at a final concentration of 10 μM and denatured at 95°C for 5 min in a PCR machine. The machine was then turned off, and the tube was cooled to room temperature over 30 min. The LA guide sequence double-stranded fragment was cloned into the pX330 vector using the following protocol (16,26): 1 μg pX330 was digested with 10 U *Bbs*I (Thermo Scientific, Waltham, MA), 2 μL 10× Buffer G in the presence of 400 U T4 ligase, 1 μL annealed oligonucleotide (10 μM stock), and 1 mM ATP at 37°C overnight. Next, 2 μL of the ligation reaction was used to transform competent cells. Positive clones were selected by *Bbs*I and *Sca*I digestion,

Table 1. Oligonucleotide sequences used in this work.

Primer and sequence	Application
mLAsgF: cacc**GCCTTTCTCGTGAACTAGTCA** mLAsgR: aaacTGACTAGTTCACGAGAAAGGC	Oligonucleotides for cloning the LA guide sequence (bold) into the pX330 vector. The sequences in lower case are the overhangs complementary to the overhangs of the cloning vector pX330 digested by *Bbs*I. The resulting clone, pX330-LAsg, used as a PCR template (Figure 1).
sgLAF: TGTAATACGACTCACTATAGGCC**TTTCTCGTGAACTAGTCA** sgRNAR: AAAAGCACCGACTCGGTGCC	Forward primer sgLAF contains a T7 promoter (underlined) and LA guide sequence (bold). Reverse primer sgRNAR primer binds in pX330. These primers were used to PCR synthesize the LAsgRNA template from pX330-LAsg for in vitro RNA transcription to produce the sgRNA (LAgRNA) for cleaving plasmid D. PCR product: 142 bp.
T3gRNAF: TGTAATACGACTCACTATAGG**CTAAATTAACCCTCACTAA**GTTTTAGAGCTAGAAATAGC sgRNAR: same as above	Forward primer T3gRNAF contains a T7 promoter (underlined), the guide sequence for the T3 promoter (bold), and the first 20 bases of the tracrRNA sequence in pX330. Reverse primer sgRNAR primer binds in pX330. These primers were used to PCR synthesize the T3 promoter sgRNA template from pX330 for in vitro RNA transcription to produce the sgRNA (T3gRNA) for cleaving the cloning vector A1. PCR product: 140 bp.
AqugblockF: ATCATTTTGGCAAAGAATTCGAACTTAGTCATGGTGTCAAAGGGTGAGG AqugblockR: TTGGTGGCCGGGAAGGG	PCR primers to amplify the insert for Gibson assembly from the gBlock fragment by adding 21 bases of left homologous sequence (underlined in AqugblockF) and with 40 bases of right homologous sequence (ttggtggccgggaaggggcacgattgtcttcactagccat; the AqugblockR sequence is underlined) already present in the gBlock fragment. PCR product: 853 bp, to be inserted into the cloning vector A1 by Gibson assembly; resulting clones: G5–8 (Figure 3 & Supplementary Table S1).

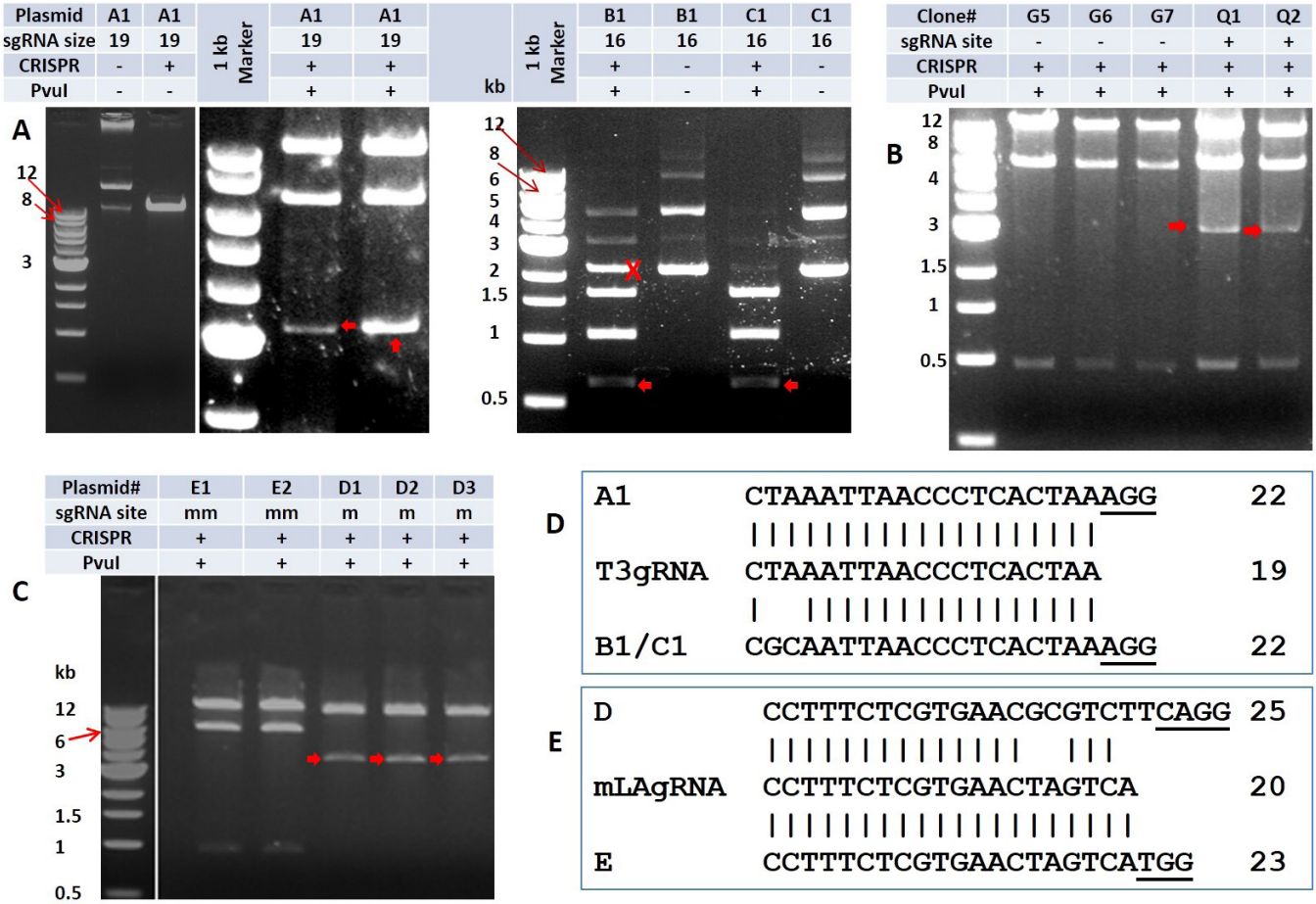

Figure 2. Cas9/sgRNA digestion specificity. (A) Cas9/T3gRNA digestion of plasmids A1, B1, and C1. The Cas9/T3gRNA digestion was 1 h for A1, and 72 h for B1 and C1. When Cas9/T3gRNA digestion was combined with restriction digestion with *PvuI*, the expected bands were produced for each plasmid (red arrows). The red X denotes a band not cleaved by Cas9/T3gRNA. (B) Cas9/sgRNA digestion requires the presence of the target sequence corresponding to the guide sequence of the sgRNA. The three positive clones (G5–G7) and two negative clones (Q1 and Q2) obtained from the CRISPR/Gibson cloning were digested with the Cas9/T3gRNA and *PvuI*. A positive clone has the insert, but the target sequence has been deleted, while a negative clone does not have the insert but still has the target sequence. (C) Cas9/LAgRNA only cleaves a vector containing a target sequence with a perfect match to the guide sequence. The target sequence of vector D has a perfect match (m, match) with the guide sequence of LAgRNA, while vector E has a target sequence with several mismatches (mm) with the guide sequence. (D) Sequence alignment of the target sequences of plasmids A1, B1 and C1 with the 19 bp T3gRNA guide sequence. (E) Sequence alignment of the target sequences of vectors D and E that are matched (m) and mismatched (mm), respectively, with the guide sequence of LAgRNA. The PAMs including the 5′-NRG are also shown underlined. The number is the length of the corresponding sequence shown in (D) & (E).

and the resulting clone was named pX330-LAsg.

b) PCR amplification of DNA templates for synthesis of sgRNAs by in vitro transcription with T7 RNA polymerase: The template for the sgRNA (T3gRNA) targeting the T3 promoter sequence in the cloning vector A1 (pLACAGRF-PTetOn; 21683 bp) was PCR amplified from pX330 using the forward primer T3gRNAF, which contained 3 parts: a T7 promoter sequence, the 19-bp guide sequence for the T3 promoter of vector A1 (position 12651–12669), and the first 20 bases of the tracrRNA sequence from the pX330 vector (16) (Addgene plasmid 42230) (Table 1). The template for the sgRNA (LAgRNA) targeting the LA sequence was PCR amplified from pX330-LAsg using the forward primer sgLAF containing the T7 promoter sequence and the guide sequence for LA. The same reverse primer (sgRNAR) binding to the sequence (position 347–328) in pX330 was used in both cases. The PCR reaction mixture contained 2 µl 10× Pfx50 PCR mix, 2.4 µl 2.5 mM dNTP mix, 1.2 µl 10 µM forward and reverse primer mix, 0.4 µl plasmid template (2.2 ng/µl), and 0.4 µl Pfx50 DNA polymerase (5 U/µl) (Life Technologies, Grand Island, NY). Sterile distilled water was added to bring the total reaction volume to 20 µl. The PCR cycling parameters were: 94°C for 2 min, 5 cycles of 94°C for 15 s and 68°C for 20 s, 5 cycles of 94°C for 15 s and 66°C for 10 s, 68°C for 20 s, and 25 cycles of 94°C for 15 s, 63°C for 10 s and 68°C for 20 s, and 1 cycle of 68°C for 10 min. PCR products were extracted with phenol/chloroform and then purified using an S-300 microSpin column (GE Healthcare Bio-Sciences, Pittsburgh, PA) following the manufacturer's instructions.

c) In vitro transcription: The in vitro transcription was conducted for 4 h at 37°C in a 50 µl reaction mixture containing 5 µl 10× RNAPol reaction buffer, 2.5 µl 10 mM NTP mix, 0.5 µl 10 mg/mL BSA, 1 µl murine RNase inhibitor (40 U/µl), 40 µl purified PCR product (17 ng/µl), and 1 µl of T7 RNA polymerase (50 U/µl). The template DNA did not

affect the subsequent Cas9 digestion, so it was not removed. The transcription product was then purified as in step b) or used directly without any obvious adverse effects.

2. Linearization of cloning vector using sgRNA and Cas9

The digestion was carried out in a 30 µl reaction mixture composed of 3 µl 10× Cas9 nuclease reaction buffer, 126 ng (300 nM) sgRNA, and 1 µl 1 µM Cas9 nuclease (NEB). Sterile distilled water was added to bring the total reaction volume to 30 µl. The final concentration of Cas9 nuclease was 30 nM. There is no unit definition for this enzyme from the manufacturer (NEB). The mixture was pre-incubated for 10 min at 37°C and then 30 nM plasmid DNA was added, and the mixture was incubated for 1 h (as recommended by the manufacturer), overnight, or 72 h, following the manufacturer's protocol. The vector A1 that had been digested overnight was used for Gibson cloning. The overnight Cas9 digested vector was purified as in step 1b.

3. Gibson cloning

A gBlock fragment containing the Aquamarine cyan fluorescent protein gene with the P2A sequence was synthesized (IDT). The insert was PCR amplified in a 20 µl reaction mixture composed of 4 µl 5× PrimeSTAR GXL buffer, 1.6 µl 2.5 mM dNTP mix, 0.4 µl 10 µM primers AqugblockF and AqugblockR, 0.25 µl gBlock template (10 ng/µl), and 0.4 µl PrimeSTAR GXL DNA polymerase (5 U/µl) (Clontech Laboratories, Mountain View, CA). Sterile distilled water was added to bring the total reaction volume to 20 µl. The PCR cycling parameters were: 94°C for 2 min, 5 cycles of 94°C for 15 s and 72°C for 20 s; 5 cycles of 94°C for 15 s and 70°C for 20 s, 26 cycles of 94°C for 15 s and 68°C for 20 s, and 1 cycle of 68°C for 10 min. The PCR-amplified fragment (853 bp) and the Cas9/sgRNA digested vector A1 were phenol/chloroform extracted and then purified by an S-300 microspin column as described above. The purified vector (63 ng) and insert (47 ng) mixture (10 µl) was mixed with 10 µl Gibson Assembly Master Mix and incubated at 37°C for 1 h. The quick and clean cloning (QC) method also was used as described previously (2): 16 µL A1 vector (7.3 ng/µl), 1.5 µL PCR product (47 ng/µl), 2 µL 10× T4 ligase buffer (NEB), and 0.5 µL T4 DNA polymerase (NEB) were mixed and incubated in a PCR block for 60 min at 15°C.

4. Transformation and selection of positive colonies

Two microliters of Gibson or QC reaction was used to transform 100 µL of home-made DH5a competent cells following a standard transformation protocol. The mini-preparation of plasmid DNAs was carried out using Zymo-Spin II columns (Zymo Research Corporation, Irvine, CA). Positive clones were identified by restriction enzyme digestion and sequencing.

Results and discussion

Since the CRISPR/Cas9 technique has been used to cleave dsDNA in vivo with high efficiency (15–25,27), the technique should be able to linearize a circular vector in vitro for cloning. Our aim was to linearize the 22 kb plasmid A1 (pLACAGRFPTetOn) at a specific site in the T3 promoter by in vitro CRISPR cleavage using Cas9 and an sgRNA (T3gRNA) targeting the T3 promoter, followed by cloning of an insert into that site by Gibson assembly.

However, CRISPR may have problems with off-target cleavage (28). Perfect base-pairing of the 10–12 bp directly 5′ of the PAM (PAM-proximal) may be sufficient to determine the specificity of Cas9 digestion (14), which could permit mismatches, especially at the 5′ end of the guide sequence. This may be a concern if a cloning vector contains one or more sequences that are slightly mismatched to the guide sequence. We therefore first examined the specificity of in vitro CRISPR cleavage using Cas9 and T3gRNA for three plasmids containing a T3 promoter. Plasmid A1 has a target sequence that is fully matched with the 19-bp T3gRNA guide sequence (Figure 2D) containing 16 bp of the T3 promoter. Plasmids B1 & C1 (2 short hairpin RNA plasmids derived from pBS/U6) have the T3 promoter sequence, which is only fully matched with the 3′ 16 bp of the 19-bp guide sequence of T3gRNA (Figure 2D). The results show that the Cas9/T3gRNA can specifically digest all clones, but only does so efficiently for plasmid A1. When combined with the restriction enzyme *Pvu*I, the correct CRISPR-digested band was present as predicted for each plasmid (Figure 2A, Supplementary Table S1). The digestion of plasmid A1 was complete after 1 h. DNA degradation or non-specific digestion was not observed after prolonged incubation (up to 72 h), indicating that the Cas9 digestion is specific and should be suitable for use in cloning. However, the digestion of plasmids B1 & C1 was not complete even after 72 h. After 1 h of digestion as recommended by the manufacturer, only a small fraction of plasmid was digested (data not shown). The sequence alignment shows that B1 or C1 only had two 5′ mismatches compared with the A1 vector (Figure 2D). This finding indicates that the Cas9/T3gRNA cleavage of the sequence with the 16-bp match to the guide sequence was less efficient than that of the sequence with the 19-bp match, and that the mismatches distal to the PAM sequence can still severely reduce the cleavage efficiency. Perhaps the mismatches thermodynamically destabilize the DNA/Cas9/sgRNA complex, which is surprising because a previous study showed that there was no obvious difference of cleavage efficiency between 17-base and 20-base sgRNAs in vivo (29). A prolonged digestion or a higher concentration of Cas9/sgRNA may be required to digest all of the B1 or C1 DNA.

The specificity of CRISPR digestion was also tested on two other plasmids (plasmids D and E) using an sgRNA (LAgRNA) targeting the LA sequence. These plasmids were constructed for a gene-targeting project and were readily available to be used for the present study. Plasmid D is a BAC subclone containing a 12.4 kb genomic DNA fragment of the mouse lipopolysaccharide-responsive beige-like anchor (*Lrba*) gene and has a target LA sequence with a perfect match to the guide sequence of LAgRNA. Plasmid E is modified from plasmid D by an insertion that interrupts the LA sequence, resulting in a target LA sequence with several mismatches to the guide sequence of LAgRNA (Figure 2E). Cas9/LAgRNA cleavage combined with *Pvu*I digestion showed that the

Cas9/LAgRNA complex cleaves only plasmid D, which has a perfect match with LA guide sequence of LAgRNA (Figure 2C).

We used Gibson assembly to clone a 783-bp insert into the Cas9/T3gRNA-linearized plasmid A1. This Gibson cloning produced 287 colonies, while the QC plate produced only a few colonies. Since the insert has one *Psp*XI site, but plasmid A1 does not, successful cloning would introduce a unique *Psp*XI cleavage site into the clones. We used *Psp*XI and *Nhe*I digestion, which produces two unique fragments (Supplementary Table S1), to identify positive clones. The four clones (G5–G8) examined from the Gibson cloning were all positive, while the four clones (Q1–Q4) examined from the QC plate were all negative (Figure 3A). This was confirmed by sequencing, which showed that the two junctions of the vector and insert were seamlessly joined (Figure 3, B and C). Cas9/T3gRNA cleavage combined with *Pvu*I digestion also distinguished positive clones from negative clones (Figure 2B). However, we observed that CRISPR-only treatment resulted in the apparent linearization of the positive clones, which should no longer have the target sequence. We believe these results suggest that Cas9 may have a topoisomerase activity that changes plasmid conformation (Supplementary Figure S1, A and B).

Gibson assembly requires an exact match of the corresponding homologous regions at the ends of the linearized vector and linear insert (5). After the ends of the Cas9/sgRNA-linearized vector have been chewed back by T5 exonuclease and annealed to the ends of the insert, there are protruding 3′ overhangs (Figure 1: step 3b) that must be removed by DNA polymerase before the DNA can be ligated. The enzymes in the Gibson Assembly Master Mix are not disclosed by the company. However, in the original protocol, the authors used Phusion DNA polymerase, which has 3′ to 5′ exonuclease activity. The Master Mix may contain the same enzyme or a similar one having 3′ to 5′ exonuclease activity to remove these heterologous regions before filling in any gaps next to the homologous regions. Therefore, the two homologous sequences are not required to be located at the very ends of the vector produced by the Cas9/sgRNA digestion, as required by the manufacturer's Gibson cloning manual. In fact, in other homologous recombination-based protocols, up to several hundred base pairs of heterologous sequences flanking the homologous sequence from both ends can be efficiently removed (2,4). In this case, the heterologous sequences at the 2 ends are 18 bp and 12 bp, and they did not affect the Gibson cloning. This property is important as it allows one to choose homologous sequences away from the Cas9/sgRNA cleaved ends of the linearized vector and to clone seamlessly using our method.

The specificity of the Cas9/sgRNA digestion is determined by the 19-bp target sequence in the vector that is complementary to the guide sequence of the sgRNA. A PAM (5′-NRG) sequence is required at the 3′ end of the target sequence. Thus, a sequence complementary to the guide sequence can be found close to a targeted site anywhere in the vector as the sequence can be in either strand. The predicted frequency of NRG in a random dsDNA sequence is 1 in every 4 bp. The sgRNA-guided Cas9 digestion is highly specific as the predicted frequency of a 16-bp gRNA sequence is 1 in every 4.3 billion bp. This is larger than the 3.3 billion-bp human genome. Although off-target sequences may have up to 5 mismatches, using a shorter guide sequence may improve specificity but may be less efficient than the 16 bp used in this study. Consistent with these results, a minimum of 17 nucleotides of complementarity are required for efficient CRISPR-Cas nuclease activity (29). We did not notice any obvious difference in digestion efficiency between the 19-base (T3gRNA) (Figure 2A) and the 20-base guide sequences (LAgRNA) (Figure 3A) used in this study. A sequence [RCGGH (R = A or G, H = T, A, C)] was found to favor Cas9 cleavage over the canonical PAM sequence of NGG (30). The importance of the PAM-proximal sequence was confirmed by the findings that the Cas9/sgRNA complex first binds to the PAM and then unwinds the adjacent DNA (30). The 2 corresponding 5-bp sequences in this study are AAGGG for the T3 sgRNA and ATGGC for the mLA sgRNA. Although they are not optimal PAM-surrounding sequences, we did not notice any digestion problems. It is unknown whether the optimal PAM-surrounding sequence role applies to the in vitro Cas9/sgRNA system.

In summary, we successfully cloned a DNA fragment into a large vector (22

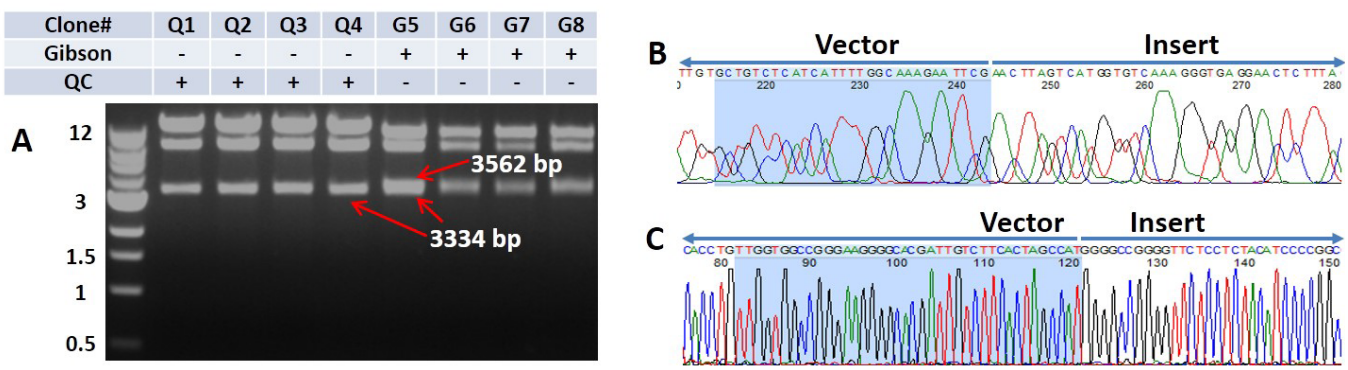

Figure 3. Gibson cloning with the Cas9/T3gRNA-linearized plasmid A1. (A) Restriction enzyme characterization of plasmid DNA extracted from four clones from the Gibson cloning and four clones from QC cloning. All clones shown were double digested with *Nhe*I/*Psp*XI. A *Psp*XI site is present in the insert but not in the vector. Clones Q1–Q4 from QC cloning are negative. Clones G5–G8 from Gibson cloning are positive, as indicated by the presence of the largest band of 9152 bp and the bottom doublet bands of 3562 bp/3334 bp. (B) Sequencing electropherograms show that the insert is correctly cloned into the vector at the 5′ end. The shaded portion is the homologous sequence in the forward PCR primer. (C) Sequencing electropherograms showing that the insert is correctly cloned into the vector at the 3′ end. The shaded portion is the homologous sequence used for the cloning.

kb) at high efficiency using the CRISPR/Cas9 nuclease combined with Gibson assembly. Our results demonstrate that the CRISPR/Cas9 technique can be used like a restriction enzyme to cleave DNA in vitro for cloning, without the limitations of the more commonly used restriction enzymes. Moreover, our cloning method does not require specific vectors or linearization by inverse PCR of a vector lacking suitable restriction enzyme sites. The methodology is quick and convenient since the whole process can be completed within 1 week, and Cas9 is commercially available. Oligonucleotides can be synthesized in 1 day, sgRNA can be synthesized in another day, and the Cas9/sgRNA digestion can be carried out overnight. Gibson assembly and transformation can be finished on the third day. Colony selection, culturing, mini-preparation of DNA, and identification can be done on the fourth and fifth days. This time frame is in sharp contrast to current protocols, which can take up to 1 month or more to obtain a positive recombinant adenoviral or baculoviral clone.

Author contributions

J.W. contributed to the conception of the study, writing and editing of the manuscript, and analysis of the experiments. A.W., K.L., B.W., S.J., and M.R. contributed to the execution of the experiments.

Acknowledgments

This work was supported by a grant from the Joy McCann Culverhouse Endowment to the Division of Allergy and Immunology, Department of Internal Medicine at the University of South Florida, Morsani College of Medicine. We would like to thank Jane Carver, Professor of Pediatrics at the University of South Florida, Morsani College of Medicine, for editing this manuscript.

Competing interests

The authors declare no competing interests.

References

1. **Li, M.Z. and S.J. Elledge.** 2007. Harnessing homologous recombination in vitro to generate recombinant DNA via SLIC. Nat. Methods *4*:251-256.
2. **Thieme, F., C. Engler, R. Kandzia, and S. Marillonnet.** 2011. Quick and clean cloning: a ligation-independent cloning strategy for selective cloning of specific PCR products from non-specific mixes. PLoS ONE *6*:e20556.
3. **Aslanidis, C. and P.J. de Jong.** 1990. Ligation-independent cloning of PCR products (LIC-PCR). Nucleic Acids Res. *18*:6069-6074.
4. **Zhang, Y., U. Werling, and W. Edelmann.** 2012. SLiCE: a novel bacterial cell extract-based DNA cloning method. Nucleic Acids Res. *40*:e55.
5. **Gibson, D.G., L. Young, R.Y. Chuang, J.C. Venter, C.A. Hutchison 3rd, and H.O. Smith.** 2009. Enzymatic assembly of DNA molecules up to several hundred kilobases. Nat. Methods *6*:343-345.
6. **de Kok, S., L.H. Stanton, T. Slaby, M. Durot, V.F. Holmes, K.G. Patel, D. Platt, E.B. Shapland, et al.** 2014. Rapid and reliable DNA assembly via ligase cycling reaction. ACS Synth Biol. *3*:97-106.
7. **Cobb, R.E., J.C. Ning, and H. Zhao.** 2014. DNA assembly techniques for next-generation combinatorial biosynthesis of natural products. J. Ind. Microbiol. Biotechnol. *41*:469-477.
8. **Taniguchi, N., S. Nakayama, T. Kawakami, and H. Murakami.** 2013. Patch cloning method for multiple site-directed and saturation mutagenesis. BMC Biotechnol. *13*:91.
9. **Hartley, J.L., G.F. Temple, and M.A. Brasch.** 2000. DNA cloning using in vitro site-specific recombination. Genome Res. *10*:1788-1795.
10. **Anderson, R.D., R.E. Haskell, H. Xia, B.J. Roessler, and B.L. Davidson.** 2000. A simple method for the rapid generation of recombinant adenovirus vectors. Gene Ther. *7*:1034-1038.
11. **Choi, E.W., D.S. Seen, Y.B. Song, H.S. Son, N.C. Jung, W.K. Huh, J.S. Hahn, K. Kim, et al.** 2012. AdHTS: a high-throughput system for generating recombinant adenoviruses. J. Biotechnol. *162*:246-252.
12. **Wiedenheft, B., S.H. Sternberg, and J.A. Doudna.** 2012. RNA-guided genetic silencing systems in bacteria and archaea. Nature *482*:331-338.
13. **Jinek, M., K. Chylinski, I. Fonfara, M. Hauer, J.A. Doudna, and E. Charpentier.** 2012. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science *337*:816-821.
14. **Hsu, P.D., D.A. Scott, J.A. Weinstein, F.A. Ran, S. Konermann, V. Agarwala, Y. Li, E.J. Fine, et al.** 2013. DNA targeting specificity of RNA-guided Cas9 nucleases. Nat. Biotechnol. *31*:827-832.
15. **Brown, A.J., D.A. Fisher, E. Kouranova, A. McCoy, K. Forbes, Y. Wu, R. Henry, D. Ji, et al.** 2013. Whole-rat conditional gene knockout via genome editing. Nat. Methods *10*:638-640.
16. **Cong, L., F.A. Ran, D. Cox, S. Lin, R. Barretto, N. Habib, P.D. Hsu, X. Wu, et al.** 2013. Multiplex genome engineering using CRISPR/Cas systems. Science *339*:819-823.
17. **Bassett, A.R., C. Tibbit, C.P. Ponting, and J.-L. Liu.** 2013. Highly Efficient Targeted Mutagenesis of Drosophila with the CRISPR/Cas9 System. Cell Rep. *4*:220-228.
18. **Friedland, A.E., Y.B. Tzur, K.M. Esvelt, M.P. Colaiacovo, G.M. Church, and J.A. Calarco.** 2013. Heritable genome editing in C. elegans via a CRISPR-Cas9 system. Nat. Methods *10*:741-743.
19. **Gratz, S.J., A.M. Cummings, J.N. Nguyen, D.C. Hamm, L.K. Donohue, M.M. Harrison, J. Wildonger, and K.M. O'Connor-Giles.** 2013. Genome Engineering of Drosophila with the CRISPR RNA-Guided Cas9 Nuclease. Genetics *194*:1029-1035.
20. **Hwang, W.Y., Y. Fu, D. Reyon, M.L. Maeder, S.Q. Tsai, J.D. Sander, R.T. Peterson, J.R.J. Yeh, and J.K. Joung.** 2013. Efficient genome editing in zebrafish using a CRISPR-Cas system. Nat. Biotechnol. *31*:227-229.
21. **Li, J.-F., J.E. Norville, J. Aach, M. McCormack, D. Zhang, J. Bush, G.M. Church, and J. Sheen.** 2013. Multiplex and homologous recombination-mediated genome editing in Arabidopsis and Nicotiana benthamiana using guide RNA and Cas9. Nat. Biotechnol. *31*:688-691.
22. **Nekrasov, V., B. Staskawicz, D. Weigel, J.D.G. Jones, and S. Kamoun.** 2013. Targeted mutagenesis in the model plant Nicotiana benthamiana using Cas9 RNA-guided endonuclease. Nat. Biotechnol. *31*:691-693.
23. **Shan, Q., Y. Wang, J. Li, Y. Zhang, K. Chen, Z. Liang, K. Zhang, J. Liu, et al.** 2013. Targeted genome modification of crop plants using a CRISPR-Cas system. Nat. Biotechnol. *31*:686-688.
24. **Wang, H., H. Yang, C.S. Shivalila, M.M. Dawlaty, A.W. Cheng, F. Zhang, and R. Jaenisch.** 2013. One-Step Generation of Mice Carrying Mutations in Multiple Genes by CRISPR/Cas-Mediated Genome Engineering. Cell *153*:910-918.
25. **Xie, K. and Y. Yang.** 2013. RNA-Guided Genome Editing in Plants Using a CRISPRCas System. Mol Plant. *6*:1975-1983.
26. **Cost, G.J.** 2007. Enzymatic ligation assisted by nucleases: simultaneous ligation and digestion promote the ordered assembly of DNA. Nat. Protoc. *2*:2198-2202.
27. **Mali, P., L. Yang, K.M. Esvelt, J. Aach, M. Guell, J.E. DiCarlo, J.E. Norville, and G.M. Church.** 2013. RNA-guided human genome engineering via Cas9. Science *339*:823-826.
28. **Fu, Y., J.A. Foden, C. Khayter, M.L. Maeder, D. Reyon, J.K. Joung, and J.D. Sander.** 2013. High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells. Nat. Biotechnol. *31*:822-826.
29. **Fu, Y., J.D. Sander, D. Reyon, V.M. Cascio, and J.K. Joung.** 2014. Improving CRISPR-Cas nuclease specificity using truncated guide RNAs. Nat. Biotechnol. *32*:279-284.
30. **Doench, J.G., E. Hartenian, D.B. Graham, Z. Tothova, M. Hegde, I. Smith, M. Sullender, B.L. Ebert, et al.** 2014. Rational design of highly active sgRNAs for CRISPR-Cas9-mediated gene inactivation. Nat Biotechnol. *32*:1262–1267.

Received 10 September 2014; accepted 08 December 2014.

Address correspondence to Jia-Wang Wang, Division of Allergy and Immunology, Department of Internal Medicine, University of South Florida Morsani College of Medicine, Tampa, FL. E-mail: jwang@health.usf.edu