# *Scarless Cas9 Assisted Recombineering (no#SCAR) in Escherichia coli, an Easy#to#Use System for Genome Editing*



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UNIT 31.8 – Current Protocols in Molecular Biology

# Scarless Cas9 Assisted Recombineering (no-SCAR) in *Escherichia coli,* an Easy to Use System for Genome Editing.

Running head: The no-SCAR system for genome editing.

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**Significance Statement**

Traditional strategies for editing the genome of *E. coli* and of other bacteria rely on two-step methods that insert a selectable marker and then remove the same marker in a subsequent step. Removal of the marker can leave scar sites that create chromosomal instability. The no-SCAR system, which is described in depth here, enables marker-free and scarless genome modifications including point mutations, deletions, and insertions. This technique enables iterative metabolic engineering in *E. coli* for a variety of applications. In addition, essential genes can be modified without the need to express them from a plasmid. According to our analysis, this is the fastest method for making multiple scarless chromosomal mutations.

**Abstract**

The discovery and development of genome editing systems that leverage the site-specific DNA endonuclease system CRISPR/Cas9 has fundamentally changed the ease and speed of genome editing in many organisms. In eukaryotes, the CRISPR/Cas9 system utilizes a "guide" RNA to enable the Cas9 nuclease to make a double strand break at a particular genome locus, which is repaired by non-homologous end joining (NHEJ) repair enzymes, often generating random mutations in the process. A specific alteration of the target genome can also be generated by supplying a DNA template *in vivo* with a desired mutation, which is incorporated by homology directed repair. However, *E. coli*, lacks robust systems for double-strand break repair. Thus, in contrast to eukaryotes, targeting *E. coli* chromosomal DNA with Cas9 causes cell death. However, Cas9-mediated killing of bacteria can be exploited to select against cells with a specified genotype within a mixed population. In combination with the well-described λ-Red system for recombination in *E. coli*, we created a highly efficient system for marker-free and scarless genome editing. This unit covers all aspects of using this method, named the no-SCAR system, for making chromosomal mutations in *E. coli*. The method consists of four basic protocols that

comprise the system, including; design of donor DNA for recombineering, identification of target sites, cloning of sgRNA for targeted counterselection, and the experimental protocol to perform genome editing.

**Keywords:** genome editing, gene deletions, λ-Red, metabolic engineering, CRISPR/Cas9, *Escherichia coli*

## Introduction

The ability to edit genomic DNA in vivo is important for many fields. Traditional methods for genome manipulations in *E. coli* rely on the insertion of selectable markers, most often antibiotic resistance genes, to enrich for the mutants while preventing growth of wild-type cells (Datsenko and Wanner, 2000). While this method is efficient, a major drawback is that the selectable marker must be removed in a second editing step. An alternative strategy for making genomic mutations relies on the incorporation of DNA oligonucleotides (oligos) at the replication fork, known as oligo recombineering (Ellis et al., 2001). Since there are no selectable makers used, it is often necessary to screen hundreds or thousands of colonies to identify cells that possess the desired mutations.

CRISPR/Cas9 is an RNA-guided DNA endonuclease that causes a double-strand break (DSB) at a targeted genomic location, known as the protospacer (Jinek et al., 2012). The well-studied *Streptococcus pyogenes* Cas9 requires a protospacer adjacent motif (PAM) of NGG on the 3' side of the target. Provided that the PAM site is present, any genomic location can be targeted by altering the cognate DNA sequence that encodes the single guide RNA (sgRNA). The sgRNA transcript interacts with Cas9 in-vivo to provide targeting specificity. Mismatches between the sgRNA and genomic target location within 12-bp of PAM site, known as the seed region, or within the PAM site itself, are generally not tolerated and abolish Cas9 activity (Jiang et al., 2013; Jinek et al., 2012). Thus, Cas9 can distinguish between genotypic populations that differ by as little as a single base pair.

By combining oligo recombination with CRISPR/Cas9, cells that possess a targeted mutation can be enriched from a mixed population by inducing DSB in cells that did not undergo recombination (Jiang et al., 2013). In *E. coli,* this method required the λ-phage "Red" recombination system (λ-Red) to enable efficient genome integration of donor DNA. The λ-Red system facilitates genomic integration of both single stranded DNA (ssDNA) and double stranded DNA (dsDNA) at the replication fork of growing cells (Maresca et al., 2010; Mosberg et al., 2010). Using this concept of counterselection against wild-type cells, we developed a plasmid-based system to edit the genome of *E. coli*, and demonstrated the ability to make point mutations, deletions, and insertions in a single step with regard to the genome manipulation (Figure 1) (Reisch and Prather, 2015). In practice, this system has been used to create point mutations, replace ribosome binding sites and promoters, insert degradation and epitope tags, and delete whole genes. Deletion of the host mismatch repair system is not required for making point mutations with a high efficiency. Both plasmids that comprise the system can be cured efficiently, resulting in host strains that are both plasmid-free and scar-free. Curing of the target-encoding plasmid enables the system to be used iteratively so that many chromosomal mutations can be made in a single cell line. Here, we detail the steps required to design and perform genome editing in *E. coli*. The workflow, outlined in Figure 2, consists of four basic protocols that describe the design of DNA for recombineering, design of target sites for Cas9 counterselection, cloning of targets into the pKDsgRNA plasmid, and genome editing.

**Basic Protocol 1**

**Title: Design of donor DNA for recombineering**

Genome editing techniques facilitated by λ-Red that use oligos as the DNA donor have been well established in literature (Ellis et al., 2001; Costantino and Court, 2003; Wang et al., 2009; Wang and Church, 2011). Several important oligo design considerations have been identified that can increase the

efficiency of recombineering, including; length of oligonucleotide, addition of phosphorothioate bonds, reducing oligo secondary structure, and targeting the lagging strand of genomic DNA. In basic protocol 1, the steps required to design oligos for recombineering with the no-SCAR system are described. Web-based computational tools such as MODEST or MERLIN are available to aid in the design of oligos and may be useful to check manual designs or for large-scale experiments, although they are not described here (Bonde et al., 2014; Quintin et al., 2016).

The design of recombineering templates and sgRNAs that target the unmodified target (basic protocol 2) are presented separately. However, it is imperative that each be performed with the other in mind to ensure that a PAM site is present within 15 bp of the desired mutation. While we have successfully made mutants that have only one bp change, in some cases a single bp change in the 12 bp seed region of the sgRNA target may not be sufficient to disrupt cutting. Consequently, additional mutations may be useful in order to disrupt Cas9 targeting. An added advantage of creating several mismatches within a small window prevents methyl directed mismatch repair (Sawitzke et al., 2011). Also, in the rare instance where there is no PAM site within 15 bp of the desired mutation, silent mutations can be added near the PAM site to disrupt *cas9* targeting.

**Materials**

DNA sequence of target regions.

*A plasmid Editor (http://biologylabs.utah.edu/jorgensen/wayned/ape/) and Benchling.com are two free and easy to use programs for DNA editing.*

DNA editing program such as benchling.com or A Plasmid Editor

1. Obtain the DNA sequence that is up- and down-stream of the mutation loci and paste into a DNA editing program.

2. Design the desired DNA sequence by inserting point mutations, removing DNA sequence, or inserting DNA sequence, to create a template that is identical to the genotype desired.

   *When creating point mutations, incorporate a C:C mismatch to evade mismatch repair in E. coli* (Costantino and Court, 2003). *Alternatively, as shown in Figure 3, create additional mismatches that result in at least three mutations within 4 bp of the desired mutation, which also evades the mismatch repair system* (Sawitzke et al., 2011).

   *Gene deletions of several hundred bp or less are very efficient. Larger deletions, up to 62 kbp have been demonstrated, but the efficiency decreases with increased size* (Wang et al., 2009). *Likewise, insertions of up to several hundred base pairs are also efficient, while insertions of several kbp are possible, they are much less efficient.*

3. Select about 80 bp of sequence that spans the desired mutations and copy the sequence that corresponds to the lagging strand in *E. coli.*

   *Use the following guide to ensure that the lagging strand is targeted;*

   - *If the target is on replichore 1 (>3923998 or <1588774) then the oligo sequence should be complementary sequence to the (+) strand sequence*
   - *If the target is on replichore 2 (> 1588774 and < 3923998) then the oligo sequence should be the same sequence as the (+) strand sequence*

   *For example, the rpsL gene is located on replichore 2 at 3.47 mbp and has its coding sequence on the negative strand. Thus, to target the lagging strand, the oligo must be complementary to the coding sequence.*

4. Check the secondary structure of the oligonucleotide with mfold (http://unafold.rna.albany.edu/?q=mfold/DNA-Folding-Form) using the default parameters.

5. If the delta G is less than -12.5 kcal $mol^{-1}$ then the oligo should be redesigned to decrease the secondary structure. The oligo can be shifted upstream or downstream so that the mutation is no

longer in the middle of the oligo, though at least 15 bp should be kept between the mutation and the end of the oligo.

6. Add 2-4 phosphorothioate bonds at the 5' end of the oligo, indicated by * for both Integrated DNA Technologies (IDT, Coralville IA) and Sigma-Aldrich (St. Louis, MO).
7. Order the oligonucleotide from your preferred supplier. Oligos from both IDT and Sigma-Aldrich have been successfully used.

   *Standard desalting is adequate for purification and the minimum synthesis scale for an 80mer is sufficient.*

   *Recombineering transformations that yield selectable mutations in the absence of Cas9 targeting may be useful positive controls for recombination. The rpsL mutation oligo designed in Figures 3 and 4 yields cells that are resistant to 50 mg $L^{-1}$ streptomycin. However, the $strep^R$ phenotype is recessive, and chromosomal copies must be fully segregated to show the $strep^r$ phenotype. Thus prolonged recovery of about 5 hours is recommended.*

**Alternate Protocol 1 - Design of double stranded DNA (dsDNA) for recombineering**

Oligos are generally considered to be the most efficient DNA donor for recombineering. However, the length of homology and size of DNA insertions is inherently limited due to the size constraints of the oligo itself. Oligos over 90 bp can be synthesized, but the cost increases significantly while quality decreases. In addition, strong secondary structure is more likely with very long oligos, which may result in decreased efficiency of incorporation. This problem can be overcome by instead using dsDNA as the DNA donor. Within the last few years the cost and delivery time of custom made dsDNA fragments has decreased significantly, specifically with the availability of gBlocks from IDT and DNA strings from Thermo-Fisher (Waltham, MA). DNA fragments of 500 bp can be designed de novo, with longer up- and downstream homology than possible using oligos. The longer homology arms increase the efficiency of

recombination, possibly negating the decrease in efficiency that results from using dsDNA as compared to ssDNA. Thus, for inserting sequence tags, modifying the 5' untranslated region, inserting multiple point mutations into a single locus, amongst other applications, we recommend synthesizing dsDNA fragments with 100-200 bp of homology up- and downstream of the target site.

**Materials**

DNA sequence of targeted regions

DNA viewing program and internet access

High fidelity DNA polymerase

Additional material and reagents for PCR amplification (UNIT 15.1) and purification of DNA (UNIT 2.1A).

1. Obtain the DNA sequence of the site of interest, including about 200 bp of up- and downstream sequence.
2. Design the insertion, deletion, or mutations.
3. Copy the DNA sequence including 100-200 bp of homology on both sides of the editing location and order dsDNA from IDT, Thermo-Fisher, or another preferred source.
4. Design and order oligos to PCR amplify the entire construct ordered above.

    *The amount of linear dsDNA provided from synthesis typically ranges from 250 ng – 1 µg. PCR amplification of the construct ensures sufficient quantities of DNA for recombineering (see step 5). To further increase efficiency of recombineering, the oligo which corresponds to the lagging strand can have phosphorothioate bonds added to the 5' end, though this is generally not required. Only the lagging strand should be modified with phosphorothioate bonds, do not add phosphorothioates to the oligo corresponding to the leading strand.*

5. PCR amplify the synthesized linear DNA using a high fidelity DNA polymerase.

   *The reaction volume can be adjusted in this step. There is no set amount of dsDNA recommended for transformation. Anywhere from 100 ng to several µg may be used. In general, more DNA is better, as long as salts or other contaminants are not present which will interfere with the electroporation in basic protocol 4.*

6. Purify the PCR product using a spin column or ethanol precipitation (UNIT 2.1A).
7. Elute or re-suspend the DNA in $dH_2O$ so that the concentration is 100-500 ng $\mu L^{-1}$ and save for use in basic protocol 4.

**Basic Protocol 2 – Identification and design of sgRNA targets**

Cas9 target specificity is determined by the 20 bp sgRNA sequence, which is encoded by a cognate 20 bp DNA sequence on plasmid pKDsgRNA-xxx. This plasmid is designed so that the transcriptional start site of the Ptet promoter is the first bp of the sgRNA target sequence. Modifying this 20 bp sequence, a process termed retargeting, is performed using ligation independent CPEC cloning or round the horn cloning with ligation (Quan and Tian, 2011; Ochman et al., 1988). The identification of Cas9 target sites and the design of the sgRNA may initially seem complicated, but it is relatively easy and scalable using the plasmids and methods described here. Design mistakes are common, time consuming, and frustrating, and we hope to limit such mistakes with this protocol. To find an appropriate target, first identify a NGG PAM site within 15 bp, and on the 3' side, of the desired mutation or deletion. The PAM site can be on either strand, though the protospacer must remain on the 5' side of the PAM (5'-$N_{20}$NGG-3', Figure 4). Here, the steps required for identification of targets, the design of sgRNA, and checking for off-target potential are detailed.

**Materials**

DNA sequence of target regions.

DNA viewing program.

1. Use the wild-type DNA sequence obtained in basic protocol 1 and identify all NGG (or CCN) PAM sites.

   *In the rpsL sequence shown in Figure 4, there are 8 NGG PAM sites. Seven sites are on the bottom strand and one is on the top strand.*

2. Select a PAM site that is within 15 bp of the mutation site and oriented so that the mutation designed in basic protocol 1 disrupts sgRNA binding by altering the PAM site or the 12 bp seed region on the 5' side of the PAM site. If the PAM site is on the top strand, then the 12 bp on the 5' side (upstream) must be modified to disrupt Cas9 targeting. If you are looking at the top strand, but the PAM site is on the bottom strand (CCN on the top strand), then the 12 bp to the 3' side (downstream) of the CCN must be modified.

   *In the rpsL example in Figure 4, there are four PAM sites within 15 bp of the point mutation, but only two are positioned on the correct side of the PAM site. PAM sites 4 and 5 are within 15 bp of the mutation site, but they are upstream of the target and mutations to alter the highlighted lysine residue will not disrupt the target site. PAM sites 6 and 7, are located on the correct side of the site so that mutations will disrupt sgRNA binding.*

   *Ideally, the target site should be between 40-60% GC and should not be <20% or >80% GC. However, the characteristics that define a good target are still poorly understood and have not been investigated in E. coli. Thus, it is likely that targets with GC content that fall outside of the optimum will still function.*

3. If the PAM site is on the top strand, copy the 20 bp DNA sequence that is immediately 5' of the PAM. If you are looking at the top strand, but the PAM site is on the bottom strand (CCN on the

top strand), copy the reverse complement of the 20 bp following of the PAM. This sequence is the target site and a part of the sgRNA-target-F primer used for retargeting the sgRNA

*Examples of sgRNA design for PAM sites on both strands are shown in Figure 4. However, the 20 bp target sequence for the top strand PAM (site #3) should not be used since the target sequence is not within 15 bp of the mutation.*

4. Add the sequence `gttttagagctagaaatagcaag` to the 3' end of the target sequence identified in step 3 to make sgRNA-target-F.
5. Take the reverse complement of the target sequence obtained in step 3 and add the sequence `gtgctcagtatctctatcactga` to the 3' end to make primer sgRNA-target-R. Order primers from a preferred supplier. Standard desalting and synthesis on the smallest scale available are sufficient.

**Find potential off-target sites**

6. Navigate to the website for "Cas-OFFinder" at http://www.rgenome.net/cas-offinder (Bae et al., 2014).
7. Select the PAM Type: SpCas9: 5'-NRG

   *The S. pyogenes Cas9 also has activity with NAG PAM sites, though it is generally weaker than NGG. Checking for off-target sites using the NRG PAM will identify both NGG and NAG sites for potential off-targeting that could decrease efficiency of genome editing. However, we recommend only using NGG PAM site when identifying targets since the activity is higher.*
8. Select the target genome; from the dropdown menu select "others" and then select *"Escherichia coli* (K-12, MG1655).
9. Paste the 20 bp target designed above into query sequence box.
10. From the dropdown menu for "Mismatch Number", select 3, and submit.

*The "mismatch", "RNA bulge", and "DNA bulge" options can be modified to make the search conditions more or less stringent. Generally, we only ensure that there are no exact matches in the 12 bp seed region. Strong off-target effects in E. coli should result in cell death, and consequently will not be observed. Though, they must be avoided because a DSB at a second site will kill cells even if the targeted site has been mutated. Weak off-target sites will most likely be repaired by homology directed repair from uncut copies of the chromosome and consequently are not a concern with this method.*

11. The results are listed in order of genomic location. Visually inspect the results to ensure that there is only one exact match to the 12 bp seed region. The target site designed above should be the only exact match, if there are additional exact matches then another target site should be chosen. You may also consider moving or redesigning the mutation site.

    *The likelihood of finding exact matches of the 12 bp seed region are very low within protein coding regions. Intergenic regions have a higher likelihood, but are still very rare.*

**Alternate Protocol 2 – sgRNA target identification using DNA2.0**

Automated design software that can identify target sites and check for potential off-target sites are available. However, bacterial genomes are rarely included in these packages. One easy to use design package that includes the *E. coli* K12 genome is provided on the website of DNA synthesis company DNA2.0 (Newark, CA) and may be useful for identifying good targets or checking manually designed targets.

**Materials**

DNA sequence of target regions.

DNA viewing program and internet access.

**Target identification and sgRNA design using DNA2.0**

1. Navigate to https://www.dna20.com/products/crispr and select "CRISPR gRNA design tool".
2. From the pulldown menu "species" select *Escherichia coli* K12 MG1655, PAM: NGG, and wild-type Cas9.
3. Input your target gene by entering either: gene name, genomic coordinates, or a user provided sequence.

   *Entering a user provided sequence enables targeting heterologous genes and checking the E. coli chromosome for off target sites.*

   *Available PAM sites and targets are ranked based on predicted off targeting. Scores of 100 have very little likelihood of having off-target affects.*
4. Download or copy the target sites that are within 15 bp of mutation designed in basic protocol 1 and orientated such that the mutation will disrupt Cas9 binding (basic protocol 2 and Figure 4).

   *To disrupt Cas9 binding, the mutations must be located within the sequence 5'-$N_{12}$NGG-3'.*
5. To design the pKDsgRNA-target-F primer required for sgRNA cloning, add the sequence "`gttttagagctagaaatagcaag`" to the 3' end of the sequence obtained from the DNA2.0 output.
6. To design the pKDsgRNA-target-R primer, take the reverse complement of the target site from step 5 and add "`gtgctcagtatctctatcactga`" to the 3' end of the sequence.
7. Order primers from preferred supplier. Standard desalting and synthesis on the smallest scale available are sufficient.

**Basic Protocol 3 - Target cloning by circular polymerase extension cloning CPEC**

The target site identified in basic protocol 2 must be cloned into the pKDsgRNA so that Cas9 will target the cognate genomic location. The DNA that encodes the sgRNA sequence is not targeted because it lacks a PAM site. One easy method to incorporate the 20 bp target is through the ligation independent cloning technique known as circular polymerase extension cloning (CPEC), shown in Figure 5 (Quan and Tian, 2011). CPEC cloning uses linear DNA fragments that are produced by PCR and possess short overlapping sequences on both ends. These overlapping regions, the sgRNA sequence in this case, can be modified by incorporating changes into the primers used for PCR amplification. The linear DNA pieces are then assembled by performing additional thermal cycling with DNA polymerase. In basic protocol 3, the details of changing the sgRNA target are described.

**Materials**

Primer gamR– `tttataacctccttagagctcga`

Target specific primers (sgRNA-target-F and sgRNA-target-R)

Primer CPEC2F – `cggcgtcacactttgctat`

pKDsgRNA-xxx (Addgene #62654 or #62656) for PCR template

Competent *E. coli* for cloning (UNIT 1.8)

*Dpn*I Restriction Enzyme

0.8% agarose gel for electrophoresis

Gel purification columns and reagents

Super Optimal Broth (SOB)

Super Optimal Broth with catabolite repression (SOC) (UNIT 1.8)

LB broth and agar plates with 50 mg $L^{-1}$ spectinomycin (UNIT 1.1)

*Taq* DNA polymerase

pKDseq5 – cagtgaatgggggtaaatgg

sgrnaR – gcctgcagtctagactcgag

sgrnaA – agctttcgctaaggatgattt

Additional reagents and equipment for agarose gel electrophoresis (Unit 2.5a), purification of DNA from agarose gels (UNIT 2.6), PCR amplification (Unit 15.1), Media Preparation and Bacteriological Tools (UNIT 1.1), Transformation of *E. coli* (UNIT 1.8), DNA sequence analysis (Chapter 7)

1. Obtain the four primers required for cloning new sgRNA targets by CPEC. Primers CPEC2F and gamR are used for all CPEC retargeting reactions. Primer pKDsgRNA-target-F and pKDsgRNA-target-R were designed in basic protocol 2.
2. Use any pKDsgRNA-xxx plasmid as DNA template and setup two 25 µL PCR reactions (primers gamR and pKDsgRNA-target-F) and (primers pKDsgRNA-target-R and CPEC2F) using a high fidelity DNA polymerase. The protocol below is recommended for Q5 or Phusion polymerase from New England Biolabs (NEB, Ipswich MA).

   1 cycle - 98° C for 2:00

   30 cycles - 98° C for 8 seconds

   64° C (-0.2/cycle) for 8 seconds

   72° C for 3 minutes
3. *Dpn*I digest the template by adding 5 units per 25 µL directly to the reaction tube and incubate at 37° C for at least 15 minutes.
4. Run the PCR on a 0.8% agarose gel and excise the bands at 3 and 4 kb, respectively. Gel purify the PCR products and elute in a minimum amount of $dH_2O$.

*Micro volume spin columns enable elution in as little as 6 µL and are available from Zymo Research (Irvine CA, Catalog #D4003) or Epoch Biosciences (Sugar Land TX, Catalog #3010-250)*

5. Perform CPEC cloning by mixing the PCR products together in a single tube and setup a PCR reaction using a high fidelity DNA polymerase. The PCR product from the last step will serve as both DNA template and primers, do not add additional template or primers.
6. Cycle the reaction as follows.

   1 cycle - 98° C for 2 minutes

   15 Cycles - 98° C for 8 seconds

   57° C for 15 seconds

   72° C for 3 minutes

*7.* Transform 5 µL of the PCR reaction into chemically competent *E. coli,* recover in 500 µL of SOC for at least one hour at 30° C, plate 200 µL on LB with 50 mg $L^{-1}$ spectinomycin, and incubate at 30° C.

*The clones grow somewhat slowly and may take 18-20 hours to appear.*

C**onfirm the sgRNA was retargeted by colony PCR and DNA sequencing**.

8. Pick an isolated colony with a sterile pipette tip and suspend in 100 µL of sterile $dH_2O$.
9. Transfer 10 µL of the cell suspension to a PCR tube and add 10 µL of 2X One*Taq* master mix (NEB) with 0.5 µM primers pKDseq5 and sgRNAR.

   *The OneTaq master mix works well and simplifies workflow, but any Taq polymerase should be sufficient.*

10. PCR amplify. The parameters below are designed for OneTaq DNA polymerase from NEB.

    1 cycle – 95° C for 2 min

    30 cycles – 95° C for 15 seconds

57° C for 15 seconds

68° C for 1 min

11. Sequence the product using the primer sgrnaA.

    *In our experience the low copy number of the pKDsgRNA plasmid makes sequencing directly from the plasmid unreliable. Sequencing the PCR product as described here gives better results.*

12. Recover the clones by in LB liquid or agar with 50 mg $mL^{-1}$ spectinomycin

    *E. coli should not be left in H20 for longer than a few hours since cell viability decreases over time.*

**Alternate protocol 3 – Round the horn cloning**

Round the horn cloning is a form of inverse PCR that can be used to perform plasmid mutagenesis by incorporating mutations into PCR primers (Ochman et al., 1988). One set of primers is used to amplify an entire plasmid followed by ligation to circularize the linear DNA (Figure 5). For re-targeting, the entire 20 bp target sequence is changed by incorporating these 20 bp onto the 3' end of the primer. This method is generally preferred since only one PCR reaction is needed. In addition, only one unique primer is required for each target since the same promoter specific primer, PtetR, can be used in every re-targeting reaction. Both Q5 polymerase (NEB) and KOD polymerase (EMD-Millipore, Billerica MA) have been successfully used for amplification of the approximately 7 kb pKDsgRNA backbone.

**Materials**

Primers – sgRNA-target-F and PtetR ($PO_4$-gtgctcagtatctctatcactga)

High fidelity DNA polymerase

0.8% agarose gel for electrophoresis

T4 DNA ligase

Additional reagents and equipment for agarose gel electrophoresis (Unit 2.5a), ligation of DNA fragments (UNIT 3.16), PCR amplification (Unit 15.1), Transformation of *E. coli* (UNIT 1.8), DNA sequence analysis (Chapter 7)

1. Obtain the two primers required for round the horn cloning of new sgRNA targets.

   *The first primer is sgRNA-target-F described in basic protocol 3. The second primer (PtetR) is the reverse complement of the 20 bp upstream of the Ptet promoter +1 site. The PtetR primer should be ordered with a 5' phosphorylation.*

2. Setup a 10-20 µL PCR reaction with a high fidelity polymerase and any pKDsgRNA-xxx plasmid as template.

   1 cycle - 98° C for 2:00

   30 cycles - 98° C for 8 seconds

   64° C (-0.2/cycle) for 8 seconds

   72° C for 3 minutes

3. *Dpn*I digest the template by adding 2 units per 10 µL to the PCR reaction and incubate at 37° C for at least 15 minutes.
4. Check the PCR product by running on a 0.8% agarose gel and ensure that the product is a single band at the correct size of 7 kb.
5. Setup a 10-20 µL ligation reaction with the PCR product at 1:20 dilution.
   a. *We typically use a 20 µL reaction setup as follows;* 16.5 µL $dH_20$, 2 µL 10X ligase buffer, 0.5 µL T4 ligase, and 1 µL of PCR reaction.
6. Incubate for at least 1 hour at room temperature.
7. Transform 5 µL into chemically competent *E. coli* and recover at 30° C in SOC for at least 1 hour.

8. Plate 200 µL onto LB agar with 50 mg $L^{-1}$ spectinomycin and incubate at 30° C overnight.
9. Screen the colonies by PCR as described in basic protocol 3.

**Basic Protocol 4 - Recombineering and Cas9 counterselection in *E. coli.***

After the recombineering DNA and counterselection plasmids have been designed and constructed, performing scarless Cas9 assisted recombineering is straightforward. The first iteration of the method, shown in Figure 1, requires the sequential transformation of plasmids pCas9cr4 and pKDsgRNA-xxx, followed by transformation of donor DNA. Here we detail the experimental steps required for transformation of the host strain, screening potential recombinants, and finally plasmid curing.

**Materials**

pCas9cr4 (Addgene #62655)

*E. coli* host strain

SOB; with 30 mg $L^{-1}$ chloramphenicol, with 30 mg $L^{-1}$ chloramphenicol and 50 mg $L^{-1}$ spectinomycin

pKDsgRNA-xxx (made in basic protocol 3)

Sterile 20% L-arabinose

Sterile 20% Glycerol – 1.5% Mannitol Solution (Chilled)

Sterile $dH_2O$ (Chilled)

Oligonucleotides or dsDNA donor

Electroporation cuvettes

Electroporator

Primers for DNA sequencing or allele specific PCR

pKDsgRNA-p15 (Addgene #62656)

Anhydrotetracycline (aTc)

LB agar plates (UNIT 1.1) containing;

30 mg $L^{-1}$ chloramphenicol

30 mg $L^{-1}$ chloramphenicol and 50 mg $L^{-1}$ spectinomycin

30 mg $L^{-1}$ chloramphenicol, 50 mg $L^{-1}$ spectinomycin, and 100 µg $L^{-1}$ aTc

50 mg $L^{-1}$ spectinomycin and 100 µg $L^{-1}$ aTc

Additional reagents and equipment for miniprep of plasmid DNA (UNIT 1.6), agarose gel electrophoresis (UNIT 2.5a), PCR amplification (UNIT 15.1), Transformation of *E. coli* (UNIT 1.8), DNA sequence analysis (Chapter 7)

1. Obtain the *E. coli* strain with plasmid pCas9cr4 from Addgene (#62655), grow overnight at 37° C in LB with 30 µg $L^{-1}$ chloramphenicol, and miniprep the pCas9cr4 plasmid.
2. Transform pCas9cr4 into the desired host strain by chemical transformation or electroporation (UNIT 1.8).
3. Recover in SOC for one hour, plate 200 µL on LB with 30 µg $L^{-1}$ chloramphenicol, and incubate at 37° C.
4. The next day, pick 1-3 colonies and prepare competent cells suitable for plasmid transformation (UNIT 1.8).
5. Transform the pKDsgRNA-xxx targeting vector made in basic protocol 2. Recover in SOC for at least one hour and plate 200 µL on LB with 30 mg $L^{-1}$ chloramphenicol, 50 mg $L^{-1}$ spectinomycin, and incubate at 30° C overnight.

   *At this point you can also plate on LB with 30 mg $L^{-1}$ chloramphenicol, 50 mg $L^{-1}$ spectinomycin, and 100 µg $L^{-1}$ aTc to test for targeting efficiency. Depending on transformation efficiency, make 3-5 tenfold dilutions and spot 8 µL of each dilution onto*

*the plates with or without aTc and incubate overnight at 30° C. If the cell killing is efficient, there will be no colonies on the aTc plate. At a minimum, there should be a tenfold change in colony counts between the induced and un-induced plates. If there is no difference in colony counts, then the targeting plasmid should be sequence verified. If the plasmid is correct, select and test a new target. Targets that have no killing are very rare in our experience.*

6. Take 1-3 colonies and inoculate into 4 mL of SOB with 30 mg $L^{-1}$ chloramphenicol and 50 mg $L^{-1}$ spectinomycin. Incubate at 30° C until the $OD_{600}$ reaches 0.4-0.5, about 4-6 hours. Induce λ-Red by addition of L-arabinose to 0.2% and incubate for 15 minutes.
7. Place the culture on ice for at least 5 minutes to quickly chill the cells.
8. Centrifuge at 3,000 x g for 10 minutes to pellet the cells and then remove the supernatant by aspiration.
9. Resuspend the cells in 1 mL of ice cold water and then gently pipette 1 mL of glycerol-mannitol solution to the bottom of the tube so that a density layer is formed with the cells layered above the glycerol mannitol solution.

    *Any method can be used to make electrocompetent cells, though we prefer the glycerol-mannitol density step method because it is quick and yields cells with consistent time constants upon electroporation* (Warren, 2011).
10. Centrifuge the cells at 3,000 x g for 10 minutes and aspirate the supernatant by first removing the top layer, and then the bottom layer of liquid.
11. Resuspend the cells in 400 µL of glycerol mannitol solution and use immediately or freeze at -80° C for later use.

    *The volumes given here can be scaled accordingly. For oligo recombineering point mutations and deletions, the volumes given above yield sufficient a number of colonies.*

**Linear DNA Transformation**

12. Take 50 µL of the competent cells prepared above and add donor DNA to a concentration of 2 µM for oligos or add 100 ng-1 µg for dsDNA. Mix gently and transfer to a 1 mm electroporation cuvette on ice.
13. Pulse using standard settings for a 1 mm cuvette of 1.8 kV, 200 Ω, and 25mF.

    *The time constant should be greater than 5 µs, though lower time constants may also yield successful recombinants.*
14. Immediately recover in 1 mL of room temperature SOC and incubate with mixing at 30° C.

    *A good control at this point is the transformation of an oligonucleotide that is not targeted by the sgRNA, and thus only cell death is expected.*
15. Perform five tenfold dilutions and spot 8 µL of each dilution onto plates with LB Cm +Spec + aTc and incubate at 30° C.

    *If the oligo incorporation and cell killing by Cas9 counterselection are efficient, then about 3 orders of magnitude more colonies should be seen on the plate with the on-target oligo, as compared to the control transformation with an off-target oligo. Lower efficiency of both killing and recombination may result in similar colony counts for both transformations. However, even in these cases it is often possible to identify mutants by screening with PCR or sequencing.*
16. Genotype the colonies on the experimental plate by Sanger sequencing (Chapter 7) or allele specific PCR (UNIT 9.8).

    *Allele specific PCR can be used for genotyping because Taq polymerases does not have 3' to 5' exonuclease activity and is unable to extend when mismatches are present at the 3' end of the primer* (Newton et al., 1989). *However, we do not recommend identification of single bp mismatches because of the tendency of Taq polymerase to extend some*

*single bp 3' mismatches* (Huang et al., 1992). *Two to four mismatches at the 3' are unlikely to extend and generally work well.*

**Target plasmid (pKDsgRNA-xxx) curing.**

17. Inoculate a single colony into LB broth and incubate at 37° C for 5-24 hours.
18. Streak for isolation on LB with 30 mg $L^{-1}$ chloramphenicol plate and incubate at 42° C overnight.
19. Patch the isolated colonies onto plates with LB only and LB with 50 mg $L^{-1}$ spectinomycin and incubate at 30° C to test for spec sensitivity.

    *The pKDsgRNA plasmid can be cured because of its temperature sensitive origin of replication. While the plasmid is unable to replicate at temperatures of 37° C or greater, the plasmid is stable at these temperatures, thus the curing occurs through dilution of rapidly dividing cells. At least one passage is generally required before cells can be considered plasmid free.*

**20.** Patched colonies that do not grow on LB with 50 mg $L^{-1}$ spectinomycin have been cured of the pKDsgRNA-xxx plasmid and can be used for subsequent steps.

**pCas9cr4 curing**

21. Transform the pKDsgRNA-p15a plasmid into the cells by electroporation or chemical transformation and recover in 1 mL of SOC at 30° C for 2-3 hours.
22. Add 100 µg $L^{-1}$ of aTc to induce *cas9* and the p15a targeting sgRNA.
23. Incubate for two hours at 30° C and then plate on LB with 50 mg $L^{-1}$ spectinomycin and 100 ng $L^{-1}$ aTc and incubate at 30° C overnight.
24. Screen 10-20 colonies by patching onto LB only and LB 30 mg $L^{-1}$ chloramphenicol and incubate at 37° C to check for the loss of chloramphenicol resistance.

*The most common problem in the curing of the pCas9cr4 is that the cells are not cured of the initial pKDsgRNA plasmid. Thus, it is important to ensure that your cells are spectinomycin$^{s}$ before transforming pKDsgRNA-p15a.*

**Commentary**

**Background Information**

The development of CRISPR/Cas9 tools has revolutionized the ability to perform targeted genome editing in eukaryotic cells. DSBs can result in functional deletions because of the error-prone nature of the non-homologous end joining (NHEJ) repair mechanism. NHEJ often creates small insertions or deletions (indels) that result in frameshift errors that disrupt gene translation. Alternatively, supplying a DNA repair template that has homology to the cut site allows for precise insertions of foreign DNA. This technology has proven remarkably versatile in terms of host range within the eukaryotes.

In bacteria, use of CRISPR/Cas9 for genome editing remains relatively uncommon (reviewed in Luo et al., 2016). In contrast to eukaryotic cells, most bacterial species do not have robust systems for NHEJ. Consequently, DSB does not cause indel formation and only results in cell death. In addition, native bacterial recombination systems are not robust enough to incorporate DNA repair templates into the genome at a high enough efficiency to carry out the gene editing techniques described in this Unit. Therefore, in *E. coli*, standard methods for performing gene deletions and insertions rely on the λ-Red system, which is highly efficient when inserting a selectable marker onto the chromosome. The λ-Red protein Beta also facilitates ssDNA recombineering, in which oligonucleotides are incorporated into the chromosome at the replication fork during DNA synthesis. Oligo recombineering is very efficient, but it inherently does not use selectable markers, which necessitates screening of many colonies to find those that have incorporated the desired mutation. Thus, it was hypothesized that combining Cas9

counterselection of wild-type cells with oligo recombineering could enable a DNA editing system in bacteria (Jiang et al., 2013; Reisch and Prather, 2015).

The use of CRISPR/Cas9 for bacterial genome editing was first demonstrated in 2013 (Jiang et al., 2013). This work demonstrated the ability to make several modifications when providing a repair template at the site of Cas9 cutting in the naturally recombinagenic bacteria *Streptococcus pneumoniae*. In *E. coli*, the work was limited to a single bp change in the *rpsL* gene using an oligo as donor DNA and recombinant expression of λ-Red. Transformation with an oligo that conferred a streptomycin$^R$ *rpsL* mutation was found in 61% of cells that were selected by resistance to CRISPR/Cas9-mediated DSBs. As a general method, however, this system was not modular because it required a strain that possessed genome integrated λ-Red. Later, the same two-plasmid system used by Jiang et al. was combined with the commonly used λ-Red plasmid pKD46 (Datsenko and Wanner, 2000; Pyne et al., 2015). In addition to oligo based recombineering, Pyne et al. demonstrated the insertion of dsDNA donors into the chromosome. Notably, this work was performed at a single genomic locus, making it unclear how robust this method is for genome engineering projects that require editing across the genome. Using similar plasmids, it was recently found that 8 of 12 targets tested in wild-type *E. coli* did not cause cell death because of inefficient DNA cutting (Cui and Bikard, 2016).

In contrast, the no-SCAR system plasmids have shown robust ability to target many different genomic locations. In fact, nine of the same targets that were used in the work by Cui and Bikard were tested for cell killing using the no-SCAR plasmids (Figure 6). Cell death was consistent for most targets, with the exception of the *eamB and treF* targets designed by Cui and Bikard. Examination of these two target sequences reveals that they have a very high GC content, and both have long stings of G's. The *treF* target has an overall GC content of 80% and 92% in the 12 bp seed region. The *eamB* target has an overall GC content of 65% and 83% in the 12 bp seed region. It has been observed that very high or low

GC content adversely affects cutting by Cas9 (Wang et al., 2014). In addition, G-rich target sites contain many PAM sites, which has also been shown to negatively affect targeting (Malina et al., 2015).

We hypothesize that the discrepancy between the 5 targets which did not function in Cui and Bikard but functioned with no-SCAR plasmids is simply the result of differences in *cas9* expression. The pCas9 plasmid used by Jiang et al., Pyne et al., and Cui and Bikard maintains the native *S. pyogenes* promoter and 5' untranslated region to drive expression of *cas9.* It is likely that the strength of this promoter in *E. coli* does not efficiently induce *cas9* expression, exacerbating the fact that some target sites are worse than others. In contrast, the pCas9cr4 plasmid has the well-tested Ptet promoter derived from the Tn10 transposon. A second key difference is that the no-SCAR systems utilizes the single guide RNA construction in which the trans-activating crRNA (tracrRNA) and target specifying CRISPR RNA (crRNA) were fused to form a chimera, thus requiring only a single transcript (Jinek et al., 2012). The system used by Cui and Bikard maintained the native dual-guide RNA system where the tracrRNA and crRNA were expressed from the native *S. pyogenes* promoter. The efficiency of these two different guide RNA systems has not been studied in *E. coli* and could contribute to the observed differences in cell death. Regardless of the mechanism, the data clearly shows that the no-SCAR system is a more robust counter-selection tool for genome editing in *E. coli*.

The data presented in Figure 6, as well as our previous work (Reisch and Prather, 2015) , was performed in wild-type *E. coli* MG1655. This is notable because many oligo recombineering methods use strains that are deficient in the methyl-directed mismatch repair system due to a *mutS* deletion. Mutation efficiency is much higher in these strains because point mutations are not reverted to wild-type. However, it is problematic that background mutations accumulate at higher rates in a *mutS* background. The effect of Δ*mutS* on genome editing efficiency with the no-SCAR system has not been investigated, but we note that the system functions well with intact *mutS*. The no-SCAR system has also

been used successfully in strain BL21 and in a Δ*recA* strain of *E. coli*. In BL21, the efficiency is lower than that observed in MG1655. For unknown reasons, λ-Red recombination efficiency in BL21 is generally lower than in K-strains, and it has been noted that multiplex automated genome engineering (MAGE) does not work in BL21 (Raman et al., 2014). Nevertheless, despite varying efficiencies, the no-SCAR system can be applied to most strains of *E. coli* without the need for modification.

The no-SCAR system works very well for the introduction of point mutations into both essential and non-essential genes. Deletions and short sequence insertions also work well, though the efficiency is generally less than that seen for point mutations. Nonetheless, screening up to tens of colonies generally enables identification of the correct mutant. Larger insertions of over 100 bp using dsDNA as template is also possible, though the efficiency is low and seems to vary widely depending on the size and location of insertion. Overall, compared to other strategies for scarless genome editing, the no-SCAR system is fast, efficient, and easy to use.

**Critical Parameters**

- Targeting
    - Be sure that the sgRNA is targeting the wild-type genome sequence.
    - Check for similar target sequences elsewhere in the genome (Basic protocol 2) and do not use targets with identical matches to the 12 bp seed region and possess a NAG or NGG PAM site.
    - Do not include the PAM site on the sgRNA template.
    - Avoid target sites with extreme GC content.
- Recombineering
    - Target the lagging strand if using an oligo as DNA template.

- The mutation must disrupt *cas9* targeting by changing the protospacer seed region or PAM site (5'-$N_{12}$NGG-3').
- Do not induce λ-Red for longer than 30 minutes because the Gam protein is toxic.

- Plasmid curing
  - Be sure that the pKDsgRNA-xxx plasmid is cured before transforming with the subsequent pKDsgRNA-xxx plasmid.

**Troubleshooting**

| Problem | Possible Cause | Solution |
|---|---|---|
| • sgRNA target cloning results in clones that possess the template target only | • Template not efficiently removed from cloning reaction | • *Dpn*I digest the PCR products<br>• Minimize the amount of template DNA in the re-targeting reaction |
| • sgRNA target is not correct | • Mutation present in oligo or incorporated during PCR | • Screen additional clones |
| • No cell death after *cas9* induction | • Improper design of sgRNA target | • Be sure that target is orientated properly relative to the PAM site and that the PAM site is not included on the sgRNA |
| | • aTc has been inactivated | • Make new plates, store in the dark |
| | • Target site does not | • Identify new target |

| | | |
|---|---|---|
| | function (very rare) | |
| • Mutants not identified | • Mutation results in a phenotype that makes cells non-viable or slow growing | • Screen colonies that appear late and grow slowly |
| | • Poor recombination efficiency of the oligo | • If possible, move the mutation and redesign the oligo<br>• Use dsDNA with extended regions of homology on either side of the desired mutation |
| • Attempting to make several mutations within a small window but mixed populations are found | • The mismatch repair system may be responsible for correcting the mutations which are not targeted by Cas9 | • Screen more colonies<br>• Perform the mutagenesis in more than one step. |
| • pCas9cr4 cannot be cured | • Cells were not cured of previous pKDsgRNA-xxx plasmid | • Ensure that the cells do not grow on LB with 50 mg $L^{-1}$ spectinomycin at 30° C before transforming pKDsgRNA-p15a |

**Anticipated Results**

**Cell death by DSB.**

Targeting the chromosome with sgRNA and *cas9* should result in cell death. As seen in Figure 6, induction of *cas9* typically results in colony counts that are $10^3$-$10^5$ lower than un-induced cells. The targets *eamB* and *treF,* which show no significant decrease in colony counts, are very rare and likely the

result of poor target selection. Another factor that sometimes leads to problems with cell death is degradation of aTc, which is light sensitive and generally labile. Plates should be stored in the dark at 4° C. If induction with aTc does not cause cell death, freshly made plates often cures this problem. Even when stored in the dark, plates older than 1 month sometimes result in poor killing.

**Recombineering and *cas9* counterselection**

The efficiency of the no-SCAR system depends on the efficiency of both λ-Red facilitated recombination and cell killing by Cas9. The parameters for oligo design described here should result in oligos that are efficient for recombination, however, we have observed that some genomic loci are more difficult to modify than others. For well-designed point mutations, it is not unusual to find that all colonies have the correct genotype. Modifications that are less efficient, such as insertions or large deletions, may require screening tens of colonies to identify one that is correct. For difficult mutations it may be prudent to use dsDNA with long regions of homology. For example, if you already have the mutation in one strain and are trying to move the mutation to a second strain, try to PCR amplify the mutation with 200-500 bp of homology on either side. Alternatively, dsDNA can be synthesized with longer regions of homology as described in alternate protocol 1.

**Time Considerations**

The design of oligonucleotides for recombineering and sgRNA targets presented in basic protocols 1 and 2 can be performed in parallel. In addition, for genome engineering projects that require several mutations, the design and cloning of sgRNA can be performed in parallel prior to beginning the in-vivo editing steps. This will decrease the lag time between performing each set of mutations.

The retargeting of sgRNA can be performed rapidly and in large batches. We have cloned up to 25 targets in one batch using round the horn cloning, described in alternate protocol 2. The PCR, ligation, and transformation can easily be performed in a single day. Generally, the clones are correct

and we proceed to the subsequent step before sequence verification is complete. However, sequence verification is recommended because clones can contain mismatches or deletions due to errors in primer synthesis.

Outside of the time required for cloning, the first iteration of the no-SCAR method can be performed in less than one week. If continuing immediately with additional mutations, subsequent iterations can be performed in as little as 3 to 4 days, assuming the pKDsgRNA-xxx and mutation oligos have already been made. As outlined previously, we argue that this is the fastest method for iterative genome engineering (Reisch and Prather, 2015).

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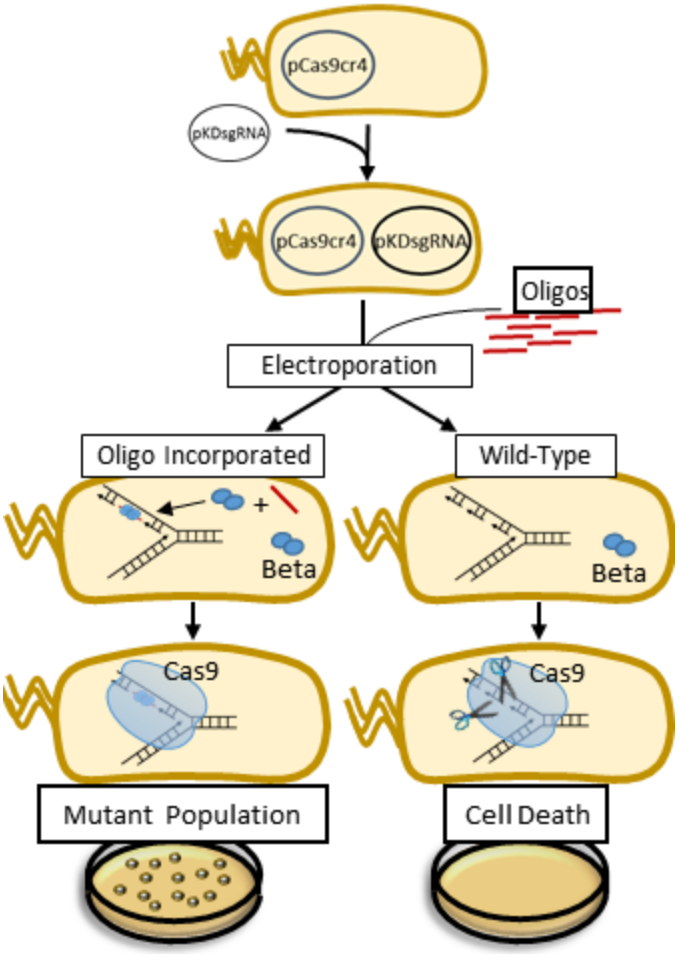


**Figure 1. Schematic of the no-SCAR system workflow**. The host strain is first transformed with pCas9cr4 and then pKDsgRNA-xxx. The pKDsgRNA plasmid possesses the λ-Red system in addition to the sgRNA that provides target specificity to Cas9. After induction of λ-Red, including Beta (blue ovals), the cells are transformed with ssDNA (or dsDNA) which can be incorporated at the replication fork of DNA synthesis. Expression of *cas9* and sgRNA that targets wild-type DNA sequence results in DSB and cell death in the wild-type cells, but not the mutant population.

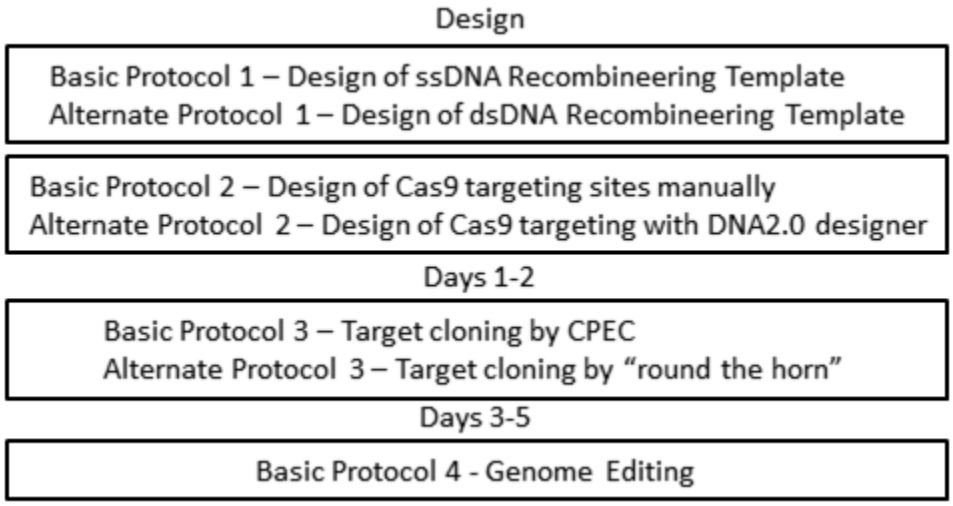


**Figure 2**. Workflow of the no-SCAR system presented in this protocol for genome editing in *E. coli*.


**Figure 3. Oligo design for recombineering of the *rpsL* gene in E. coli**. A) The up- and downstream nucleotide sequence of *rpsL* with the site of mutation highlighted in blue. B) A single bp mutation shown in red causes the substitution K43R. The 80 bp of DNA sequence corresponding to the mutation oligo is highlighted. C) Three silent mutations are shown in red to evade the mismatch repair system. D) Final oligonucleotide design that includes 4 bp mismatches shown in lowercase, 4 phosphorothioate bonds at the 5' end, and targets the lagging strand.

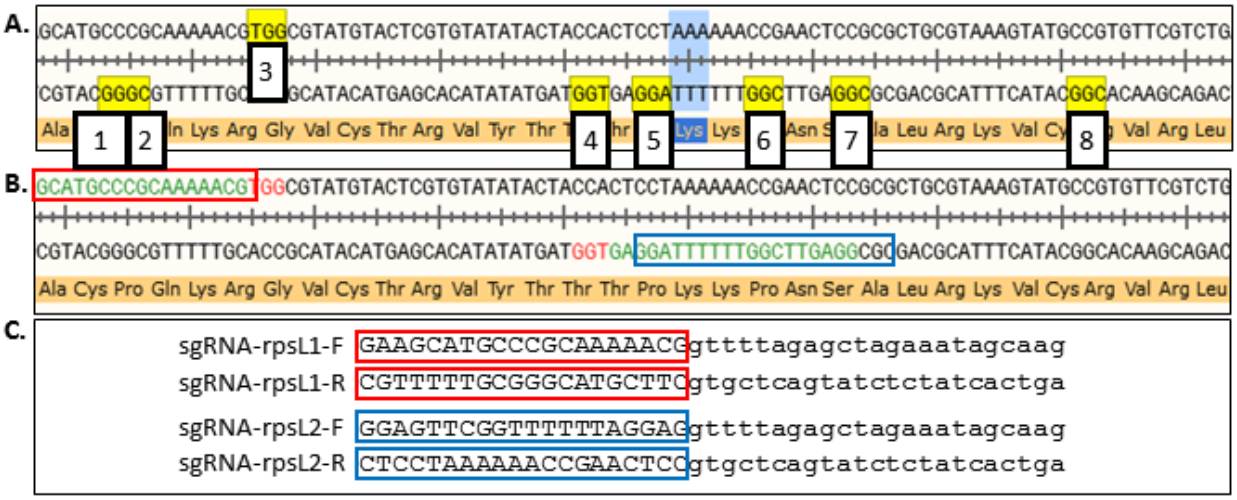


**Figure 4. Schematic of sgRNA targeting design in the rpsL gene.** A) PAM sites are highlighted in yellow and the site of point mutation is highlighted in blue. B) One PAM site on each strand is shown in red with the corresponding protospacer (sgRNA target) sequence in green and boxed. C) Primer designs for CPEC cloning of sgRNA to target the protospacer sequences shown in B.

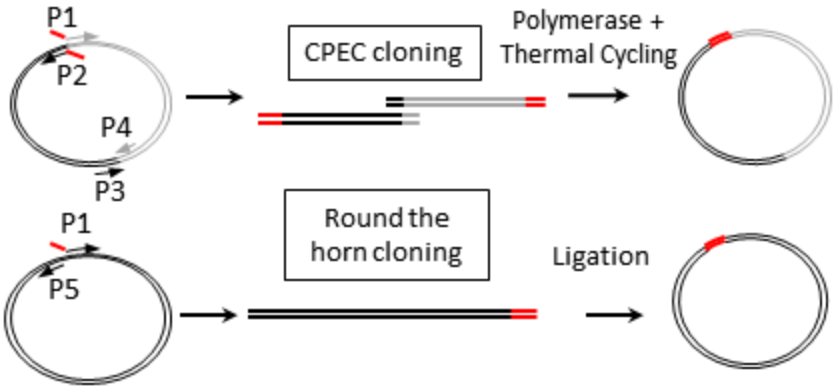


**Figure 5. Schematic for re-targeting the pKDsgRNA plasmid by two piece CPEC or round the horn cloning**. The new 20 bp target sequence, shown in red, is incorporated into primers P1 and P2. Primers; P1 – pKDsgRNA-taget-F, P2- pKDsgRNA-target-R, P3 – BetaR, P4 – CPEC2F, and P5 – pTetR(phosphorylated).

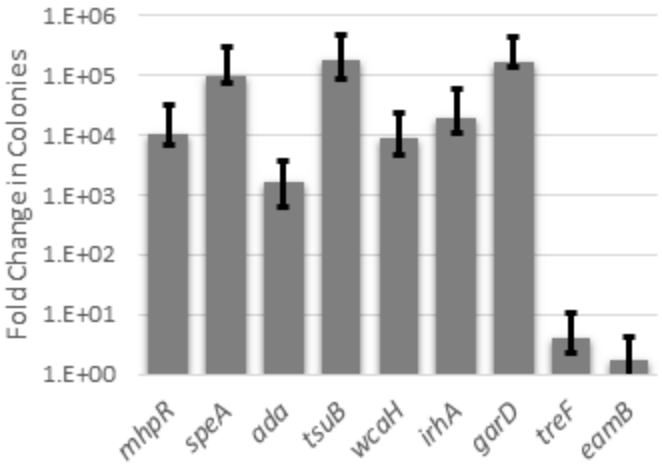


**Figure 6. Fold-change in colony counts between cas9 induced and un-induced plates for the no-SCAR system plasmids.** Cells were first transformed with pCas9cr4 and then the pKDsgRNA plasmid with each target indicated above. Single colonies were then suspended in water and six tenfold dilutions were spotted on plates with 30 mg $L^{-1}$ chloramphenicol, 50 mg $L^{-1}$ spectinomycin, and with or without 100 µg $L^{-1}$ aTc. The number of colonies on plates without aTc were divided by the number of colonies with aTc to calculate the fold-change. The fold-change given is the average of plating three isolated colonies and the error bars represent the range of the same three experiments. In the manuscript by Cui and Bikard, targets *mhpR* and *speA* caused cell death, while the remaining seven targets did not.