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Product Information

Cas9 Lambda Red and gRNA Plasmids
For CRISPR-Mediated Recombineering in *E. coli*

Cas9 Lambda Red Homologous Recombination Plasmid for *E. coli*
Catalog Number **CAS9BAC1P**

Custom CRISPR HR gRNA Plasmid
Catalog Number **CRISPRBACD**

Storage Temperature –20 °C

CRISPR Non-Target Negative Control Plasmid for Bacteria
Catalog Number **CRISPR31**

CRISPR *LacZ* Positive Control Plasmid for Bacteria
Catalog Number **CRISPR30**

TECHNICAL BULLETIN

Product Description

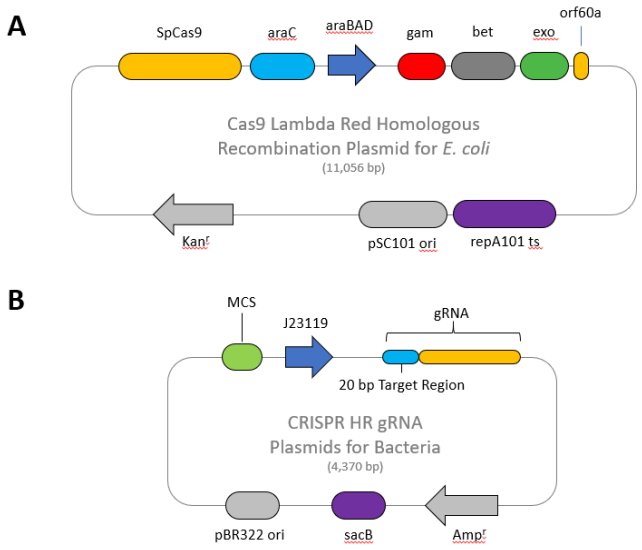
These plasmids constitute a two-part system for CRISPR-mediated recombineering in *Escherichia coli* (*E. coli*).

The Cas9 Lambda Red Homologous Recombination Plasmid for *E. coli* (Catalog Number CAS9BAC1P) contains the gene for Cas9 from *Streptococcus pyogenes* (spCas9) expressed from its native promoter, as well as the genes for λ-red recombinases exo, beta, and gam under the control of the arabinose-inducible ParaB promoter. This plasmid confers kanamycin resistance and possesses the repA101ts temperature-sensitive origin of replication, allowing for easy plasmid maintenance and curing (see Figure 1A).

- The Custom CRISPR HR gRNA Plasmid (Catalog Number CRISPRBACD) is a custom plasmid containing the spCas9 gRNA with a custom-designed spacer sequence specific to the gene of interest.
- The CRISPR *LacZ* Positive Control Plasmid for Bacteria (Catalog Number CRISPR30) contains a gRNA spacer targeting the *lacZ* gene in wild-type *E. coli*.
- The CRISPR Non-Target Negative Control Plasmid for Bacteria (Catalog Number CRISPR31) contains a non-targeting spacer.

All gRNAs are expressed constitutively from a J23119 promoter. These plasmids also contain an ampicillin resistance marker, a pBR322 origin of replication, and a *sacB* gene from *Bacillus subtilis* for counter-selection-based curing (see Figure 1B).

Figure 1.
Bacterial CRISPR HR Vector Constructs



(A) Cas9 Lambda Red Homologous Recombination Plasmid for *E. coli* Vector Map. (B) CRISPR HR gRNA Plasmids for Bacteria. The vector map shown in B illustrates the structure of all described gRNA plasmids, including the Custom CRISPR HR gRNA Plasmid, CRISPR Non-Target Negative Control Plasmid for Bacteria, and CRISPR LacZ Positive Control Plasmid for Bacteria. These gRNA plasmids vary only in the sequence of the 20 bp target region of the gRNA.

Background Information

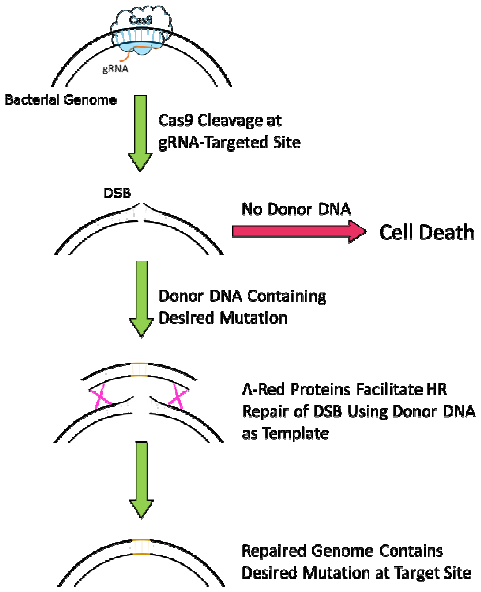
Microbial genome engineering is used for many biotechnological and biomedical purposes, including metabolic engineering, strain optimization, and general molecular biology research. One of the most commonly engineered genomes is that of *Escherichia coli* (*E. coli*).[1] Our CRISPR/Cas9-mediated λ-Red vector system offers a simple, quick method for markerless and scarless gene editing that is significantly more efficient and amenable to multiplexing than traditional methods.

E. coli has an active endogenous homologous recombination (HR) repair system, so chromosomal integration can be achieved simply by donating DNAs with long (~1,000 bp) homology arms to recombine at the target insertion site. Unfortunately, this method is very inefficient.[2] Recombination-mediated genetic engineering, or recombineering, is a popular method that utilizes phage-derived proteins to assist HR and increase the efficiency of chromosomal integration.[3]

The CRISPR system is a bacterial immune system that has been exploited by researchers as a revolutionary gene editing tool. In this system, a CRISPR-associated (Cas) endonuclease is guided by a gRNA to a specific DNA sequence, where it creates a double-stranded break (DSB).[6]

Until now, this nuclease-based method has been mostly overlooked as a microbial gene editing tool because most bacteria lack DSB repair mechanisms, rendering the technology toxic.[7] However, when CRISPR/Cas9 is used to mediate recombineering, this cytotoxic quality offers an advantage: Cas9-induced DSBs kill those cells that fail to recombine with the donor DNA, avoiding the need for selectable markers to select edited cells[5] (see Figure 2).

Figure 2.
CRISPR-Mediated Recombineering Eliminates the Need for Selection.



Recent publications using CRISPR/Cas9-mediated recombineering in *E. coli* tout editing efficiencies near 100%,[5] making CRISPR/Cas9-mediated recombineering the most powerful bacterial genome engineering method to date. In addition, Cas9-mediated recombineering overcomes the dependence on a second recombination step, avoids the creation of destabilizing scar sites, can be used in multiplexing, and is less time-consuming than previous procedures.[8]

Here we present a novel dual-vector CRISPR/Cas-mediated λ-Red system for improved recombineering in *E. coli*. This system is shown to facilitate homology-directed repair of DSBs created by the Cas9 endonuclease, enabling genetic alterations through chromosomal integration of a donor DNA. These vectors have been tested in multiple strains of *E. coli*, and have been shown to yield insertions, deletions, and point mutations, as well as multi-gene manipulations, all with high efficiency. This donor can be either ssDNA or dsDNA with homology arms of 45–50 or 150–500 nucleotides, respectively. For more information about donor design, please see the corresponding donor design sections in the procedure.

Procedure Overview
In general, the steps required for successful engineering of *E. coli* using this system are as follows:

1. Design and order custom gRNA plasmid.
2. Design custom donor DNA template(s); order or PCR amplify design(s).
3. Prepare *E. coli* strain of interest via electroporation of the Cas9 Lambda Red Homologous Recombination Plasmid for *E. coli*.
4. Electroporate *E. coli* strain from step 3 with gRNA plasmid and donor DNA.
5. Harvest genomic DNA or perform colony PCR and assay mutations.

Note: The provided procedure uses electroporation for transformation of *E. coli*. Other methods of transformation may be used, if preferred.

Components
Product Format
One vial containing 1 µg of plasmid DNA in Tris-EDTA (TE) buffer at a concentration of 20 ng/µl.

Note: The Custom CRISPR HR gRNA Plasmid (Catalog Number CRISPRBACD) is configurable and may be ordered in quantities larger than 1 µg.

Reagents and Equipment Recommended but Not Provided.
- Custom oligonucleotides from Sigma-Aldrich for PCR primers and ssDNA donor templates
- Electroporation System for Bacterial Cells
- SOC medium (Catalog Number S1797)
- LB Agar Plates with Kanamycin
- LB Agar Plates with 100 µg/ml Carbenicillin, 60 µg/ml X-Gal, and 0.3 mM IPTG
- L-(+)-Arabinose (Catalog Number A3256)
- LB Agar Plates with 5% Sucrose
- GenElute™ Bacterial Genomic DNA Miniprep Kit (Catalog Number G1N70)
- JumpStart™ REDTaq® ReadyMix™ (Catalog Number P0982) for colony PCR
- JumpStart™ REDAccuTaq® LA DNA Polymerase (Catalog Number D1313) for amplification of dsDNA donor templates
- Water, PCR Reagent (Catalog Number W1754)
- Gel Loading Buffer (Catalog Number G2526)
- Tris-Borate EDTA Buffer, 5× concentrate, powder blend (Catalog Number T3913)
- Ethidium Bromide Solution, 10 mg/ml in water (Catalog Number E1510)

Precautions and Disclaimer
For Research Use Only. Not for use in diagnostic procedures. Please consult the Safety Data Sheet for information regarding hazards and safe handling practices.

Storage/Stability
These plasmids are shipped as frozen stocks on dry ice. Once received, it is recommended the plasmids are stored at –20 °C. Avoid repeated freezing and thawing.

Procedures
The following procedures provide general guidelines for designing and ordering custom gRNA plasmids and donor template DNAs for the target of interest, as well as a detailed account of the *LacZ* control experiment for Cas9-mediated recombineering using this system.

Note: For the LacZ positive control experiment, one will need to order the recommended ssDNA donor template (see Figure 3).

Custom gRNA Plasmid Design
To design and order custom gRNA plasmid(s) for the target(s) of interest, please go to the CRISPR Design Tools page at **milliporesigmabioinfo.com**.

1. If you need to design a gRNA based on your target gene, use the CRISPR design tool and follow the prompts to design your gRNA sequence(s). If you already know your gRNA sequence, you can input this sequence directly via the same tool.

2. When prompted, select the CRISPRBACD J23119-gRNA (bacterial plasmid). Once you have configured your custom gRNA vectors(s), submit them to be redirected to the ordering page.

3. For the positive and negative control experiments, one will also need to order the corresponding positive control gRNA plasmid (Catalog Number CRISPR30) and negative control gRNA plasmid (Catalog Number CRISPR31).

Single-Stranded DNA (ssDNA) Donor Template Design

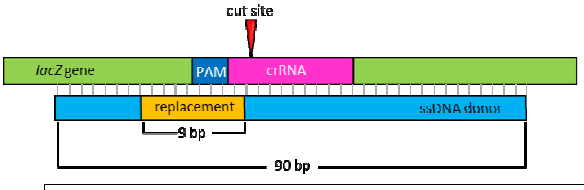
We recommend using single-stranded DNA donors when the desired mutation is a small deletion (<1 kb) or replacement (<20 bp).[5] For insertions, as well as larger deletions and replacements, we recommend using dsDNA donors, which are covered in the next section of this procedure.

1. Once you have designed your gRNA and have determined the cut site, design your ssDNA donor template using the following guidelines:
   - The donor should consist of ~90 nucleotides surrounding the cut site, altering bases as necessary to incorporate your desired mutation.
   - The resulting mutation must remove or mutate the PAM sequence or the protospacer sequence proximal to the PAM, as this prevents toxicity from repeated Cas9 cutting.[7]

   Note: We recommend trying donors that are homologous to both the sense and antisense strands, as one is typically more efficient than the other, depending on the location of the target in the chromosome.

2. For the positive control experiment, you will need to order the control ssDNA donor template. This 90-nt oligonucleotide contains a 9 bp replacement that introduces two premature stop codons in *lacZ* in wild-type *E. coli*, effectively knocking out *lacZ* function upon recombination at the target site.[5] The donor design scheme and sequence are shown in Figure 3, top right.

Figure 3.
LacZ 9 bp Replacement ssDNA Donor Design and Sequence



***lacZ* Positive Control ssDNA Donor Sequence**
C*C*CATTACGGTCAATCCGCCGTTTGTTCCCACGGA GAATCCGTGAACCTGATACTCGCTCACATTTAATGTT GATGAAAGCTGGCTA*C*A
*denotes phosphorothioate linkage

3. Order your synthetic ssDNA donor design(s) through Sigma-Aldrich at **sigmaaldrich.com/oligos**. We recommend adding phosphorothioate linkages to the 5′ and 3′ termini to protect the ssDNA from nuclease degradation upon transformation.

Double-Stranded DNA (dsDNA) Donor Template Design

For larger deletions (>1 kb), replacements (>20 bp), and insertions, we recommend using a double-stranded DNA donor. Cas9-mediated λ-red recombineering has been shown to successfully delete regions up to 12 kb using 500-bp homology arms.[5]

1. Once you have designed your gRNA and have determined the cut site, design your dsDNA donor template using the following guidelines:
   - The donor should consist of the desired mutation surrounded by homology arms on each side. These arms are homologous to the target site, which enables recombination between the genomic target and the dsDNA donor.
   - For most experiments, we recommend 300–500-bp homology arms; though, arms as short as 150 bp may be sufficient in many cases. We recommend trying donors of varying sizes for each of your target mutations and strains of interest.
   - The resulting mutation must remove or mutate the PAM sequence or the protospacer sequence proximal to the PAM, as this prevents toxicity from repeated Cas9 cutting.[7]

2. You may order these dsDNA donors as synthetic gene fragments. Alternatively, you can construct your dsDNA donors using fusion PCR. This involves separately PCR-amplifying each homology arm (and the sequence to be inserted, if applicable) from your target strain's genomic DNA using primers that create overlaps between the two fragments, then fusing these arms together as one product in a subsequent round of PCR.[9] For this step, we recommend using KOD Hot Start DNA Polymerase (Catalog Number 71086).

Preparation of *E. coli* Strain

We recommend transforming your *E. coli* strain of interest in two steps. In this first step, you will transform your strain with the Cas9 plasmid. We recommend saving this strain as a glycerol stock for future experiments. In a later step, you will transform this strain with your gRNA plasmids and corresponding donor DNAs.

1. Transform the Cas9 Lambda Red Homologous Recombination Plasmid for *E. coli* via electroporation, using a procedure that is optimal for your strain(s) of interest.

2. To select for positive colonies, spread 100 µl on LB agar plate(s) with 50 µg/ml kanamycin. Incubate for 8–12 hours at 30 °C.

3. Once colonies appear, pick one and inoculate 3 ml of liquid LB with 50 µg/ml kanamycin in a 15-ml culture tube. Incubate for 8–12 hours at 30 °C with shaking (250 rpm).

4. Save this culture as a 15% glycerol stock and store at –80 °C for future experiments.

Note: For the *lacZ* control experiment, you will need to use a strain of *E. coli* with a wild-type *lacZ* gene to enable gene knockout and blue-white screening. This typically excludes cloning strains. We recommend wild-type *E. coli MG1655* or *E. coli W3110*.

Gene Editing of *E. coli* (*LacZ* Positive Control Experiment)

In this second step, you will induce λ-red activity and transform your strain with your custom gRNA plasmid(s) and corresponding donor DNA(s). These steps detail this procedure for the *lacZ* control experiment.

1. The evening (or 8–12 hours) before the experiment, inoculate 5 ml of liquid LB with 50 µg/ml kanamycin in a 15-ml culture tube using the glycerol stock from the previous step.

2. The next morning, dilute the overnight culture 1:100 in 15-ml culture tubes with 3 ml of fresh liquid LB with 50 µg/ml kanamycin. Be sure to inoculate enough cultures to dedicate 3 ml to each experimental condition. Grow at 30 °C with shaking (250 rpm) until the OD_{600} reaches 0.3–0.5 (~3 hours).

3. Once the OD_{600} reaches 0.3–0.5, induce λ-red expression by adding a sterile arabinose solution to give a final arabinose concentration between 3 mg/ml (0.3% w/v) and 20 mg/ml (2% w/v). Incubate for one more hour at 30 °C with shaking.

4. For the *lacZ* control experiment, prepare 3 vials of electrocompetent cells, concentrating each final cell pellet into 40 µl of sterile 10% glycerol. Label these 3 tubes as L, K, and E for Lawn (negative control), Killing, and Editing, respectively.

   Note: Use your own optimized electroporation procedure for your strain of interest. This procedure is recommended for wild-type *E. coli MG1655* or *W3110* strains using 1 mm cuvettes. The final total volume should be 50 µl.

5. Electroporate the following components into each corresponding vial of cells (see Table 1), using an optimized procedure for your strain of interest:

Table 1.
LacZ Control Experimental Conditions

Rxn	gRNA Plasmid	Donor
L	60 - 100 ng of CRISPR Non-Target Negative Control Plasmid for Bacteria	None
K	60 - 100 ng of CRISPR *LacZ* Positive Control Plasmid for Bacteria	None
E	60 - 100 ng of CRISPR *LacZ* Positive Control Plasmid for Bacteria	5 µl of 10 µM *lacZ* Positive Control ssDNA Donor (1 µM final concentration)

6. Add 950 µl of pre-warmed SOC medium immediately after electroporation and incubate the outgrowth at 30 °C with shaking (250 rpm) for at least 1 hour.

7. Spread outgrowth cultures onto individual LB agar plates with 100 µg/ml carbenicillin, X-Gal, and IPTG. Initially, spread 100 µl of outgrowth directly. For L, spreading 100 µl of 10^{-3} dilution may be required.

8. Incubate at 30 °C for 16–24 hours to observe colony formation. Expected results are provided in Table 2.

Table 2.
LacZ Control Experimental Conditions

Rxn	Expected Result	Appearance
L	Non-targeting gRNA does not target the genome; therefore, Cas9 does not cut. *lacZ* gene is functional.	Lawn of blue colonies.
K	Guided by the *lacZ*-targeting positive control gRNA, Cas9 cuts at *lacZ*. Lack of donor prevents repair by HR, leading to cell death.	Killing; significantly fewer colonies than plate L. Colonies can be blue or white.
E	Guided by the *lacZ*-targeting positive control gRNA, Cas9 cuts at *lacZ*. HR with positive control ssDNA donor knocks out *lacZ* function via 9-bp replacement.	Editing; more colonies than plate K. Colonies should be mostly white.

Mutation Detection

There are many methods to detect mutations produced by CRISPR systems. Mutations resulting in phenotypic changes are the most easily screened (e.g., mutations in *lacZ* can be detected via blue-white screening). For genotyping, we recommend designing primers around the target site in the organism's genomic DNA for PCR amplification of the mutated region. It is important to make sure these primers fall outside the donor DNA sequence to prevent false positives. We recommend performing this PCR on isolated genomic DNA, though colony PCR may be sufficient in many cases. If the mutation is an insertion or deletion of significant size, gel electrophoresis of the PCR amplicon may be used to observe this change. For easy colony PCR, we recommend using JumpStart REDTaq ReadyMix (Catalog Number P0982), which enables samples to be loaded directly into an agarose gel without supplementary loading dye. If the mutation does not result in an appropriate size difference in the amplicon, we recommend sequencing the PCR product.

Note: For the positive control experiment, the following primers may be used to verify the 9-bp replacement at *lacZ* following blue-white screening. We recommend performing colony PCR and subsequent Sanger sequencing. The same primers may be used for both steps. These primer sequences are provided in Table 3.

Table 3.
LacZ Control Experiment Sequencing Primers

Primer	Sequence
lacZ-Forward	GTGAAAAGAAAAACCACCCTGGC
lacZ-Reverse	TGTTACCCGTAGGTAGTCACGCAACT

Plasmid Curing

Upon obtaining a correct clone, the gRNA plasmids can be cured by SacB-sucrose based counter-selection and the Cas9 Lambda Red Homologous Recombination Plasmid for *E. coli* by incubating the clone at 37 °C. To remove both simultaneously, follow these steps:

1. Spread a 200 µl dilution (10^{-1}, 10^{-2}, or 10^{-3}) of the selected clone on an LB agar plate with 5% sucrose (Teknova, Catalog Number L5110) and incubate at 37 °C overnight (or for 8–12 hours).

2. The next day, pick 8–16 large colonies and streak each onto 3 different plates: LB agar, LB agar with 100 µg/ml ampicillin or carbenicillin, and LB agar with 50 µg/ml kanamycin. Incubate these plates at 37 °C overnight.

3. Observe the plates. Those clones that are sensitive to both ampicillin (or carbenicillin) and kanamycin have been cured of both editing plasmids. Save the successfully cured plasmids from the LB agar plate as glycerol stocks, if desired.

References

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Troubleshooting
If no cutting is observed and there is reason to suspect an experimental flaw is at fault, the following considerations may aid the researcher in troubleshooting the experiment.

Suspected Issue	**Solution**
One of the plasmids has been thawed and refrozen too many times.	Our plasmid DNA has been shown to withstand some freezing and thawing, but aliquoting each into smaller quantities will allow this potential issue to be avoided.
The electroporation or other transformation method is not working or is too toxic.	For any transformation method, the procedure should be optimized for each strain used. For troubled electroporations, refer to the manufacturer's procedure for further assistance. For the positive control experiment in *E. coli MG1655* or *E. coli W3110*, we used the Gene Pulser® XCell™ device (BioRad, Catalog Number 165266) with Gene Pulser Electroporation Cuvettes, 0.1 cm gap (BioRad, Catalog Number 1652083). To electroporate, we used the pre-set program for *E. coli* in 0.1 cm cuvettes found on this device.
Blue and white colonies are seen on the "killing" control plate in the *lacZ* control experiment.	While most Cas9-generated DSBs will lead to cell death without a donor template for repair, some escaper colonies are to be expected, and are not necessarily an indicator of a larger problem. Blue colonies are often escapers of the CRISPR system, most commonly due to spontaneous mutations in the colony's plasmid copy of Cas9. White colonies often indicate mutations in the target sequence (*lacZ*), possibly due to very low levels of naturally occurring NHEJ repair.
The sequencing primers generate no PCR product after gene editing.	If no PCR product is observed, it is possible that the corresponding genomic binding sites are no longer present. This may be due to low levels of repair via the *E. coli* alternative end-joining pathway (A-EJ), which creates large genomic rearrangements/deletions (rather than precise changes like those generated via HR).[10] We recommend double-checking your primer sequences and optimizing your PCR reagents and methods; however, if a deletion is still suspected, you may choose to design alternate primers that bind farther from the target site.

US Patents Pending

License Agreements

CRISPR Use License Agreement

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