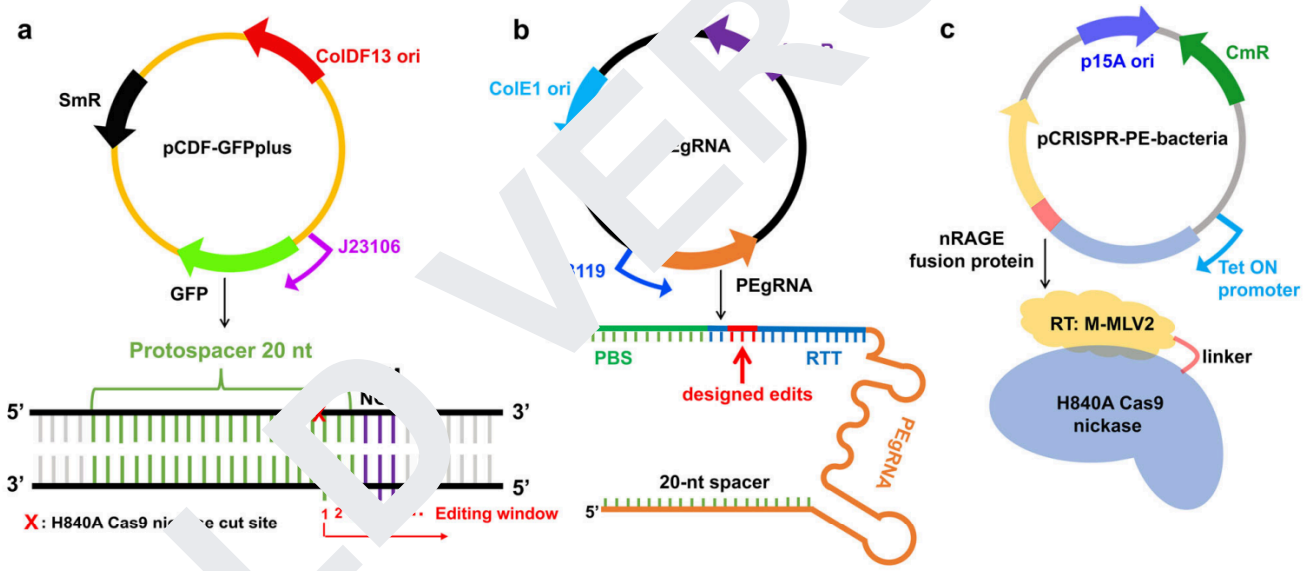
CRISPR Prime Editing in *E. coli*

Overview | Objective

In this **3-day laboratory**, we will explore the cutting-edge **CRISPR-prime e ing sy m**, a powerful tool for genetic engineering that enables insertions, deletions, a s itu s in target DNA sequences without introducing double-strand breaks. Particu ly, this xperiment will demonstrate how to practically perform gene deletion using CRISP -pr editin n *E. coli* which can be broadly applied to other designs for various applications. This ten oad applications in both basic research and biotechnology, including gene th py, meta lic engineering, and the development of novel organisms with unique characteristics.



CRISPR Prime Editing Plasmids
ource: https://www.nature.com/articles/s41467-021-25541-3#Sec22

Overview | Concepts Learned & Skills Gained

We will employ a three-plasmid system to perform the prime editing in *E. coli*. The plasmids involved are **pCDF-GFPplus**, **pPEgRNA**, and **pCRISPR-PE**. The pCDF-GFPplus serves as the reporter plasmid, containing the green fluorescent protein (GFP) gene. This gene is under the control of a constitutive promoter, which allows for continuous expression of the GFP. As a result, *E. coli* cells harboring this plasmid will exhibit fluorescence, which can be detected using the plate reader.

The pPEgRN plasmid is responsible for expressing the prime editing guide RNA (PEgRNA). The PEgRNA is designed to target the GFP gene on the pCDF-GFPplus plasmid and contains a spacer sequence, a **primer binding site (PBS)**, and a **reverse transcription template (RTT)** that

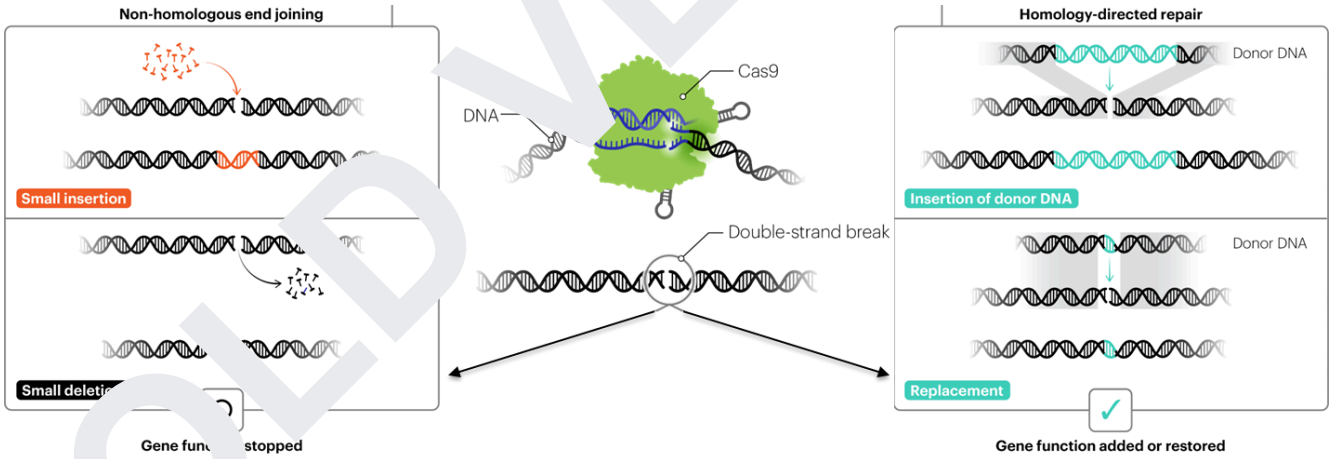
incorporates the desired edit. By designing different PEgRNAs, we can achieve various types of genetic modifications, such as insertions, deletions, or substitutions.

Lastly, the pCRISPR-PE plasmid encodes an *E. coli* codon-optimized fusion protein, which consists of the **Cas9 nickase (Cas9n)** a modified version of the Cas9 protein which "nicks" and only creates a single-strand break in the DNA instead of double-strand break. The plasmid also includes the **reverse transcriptase M-MLV2**. This fusion protein works with the PEgRNA to write the desired edit into the GFP gene. Therefore, the Cas9n introduces a nick in the target DNA strand, while the M-MLV2 reverse transcriptase copies the edited sequence from the PEgRNA into the target DNA.

The successful editing of the GFP gene will result in the introduction of a premature **stop codon**, leading to a non-functional GFP protein. As a consequence, the edited *E. coli* cells will lose their fluorescence, providing an easy-to-observe readout for successful gene editing events.

Pre-Lab | What is CRISPR-Cas9?

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) is a gene-editing tool that allows scientists to modify an organism's DNA. The system is based on a naturally occurring defense mechanism found in bacteria to fight viral invaders by cutting their DNA.



Source: Figure adapted from CRISPRpedia | Innovative Genomics Institute

The CRISPR-Cas9 system consists of two main components: the **Cas9 protein**, which acts as a molecular scissor, and a **guide RNA (gRNA)** that directs the Cas9 protein to a specific target sequence in the DNA. When the Cas9 protein and the gRNA are introduced into a cell, the gRNA binds to the target DNA sequence, guiding the Cas9 protein to make a precise cut in the DNA, technically referred to as DNA double-strand break (DSB) later. Once the DNA is cut, the cell's own repair machinery attempts to fix the break, which can result in the inactivation of a gene (see Non-homologous end joining in the figure).

While CRISPR-Cas9 is a powerful tool, it can sometimes cause unintended changes to the DNA (**off-target effects**) and is limited in its ability to introduce specific edits, like precise DNA insertions or specific point mutations. The CRISPR prime editing system, which you'll be working with in this lab, is an advanced version of CRISPR technology that addresses some of these limitations and allows for **even more precise gene editing**.

Check this video for thorough description of CRISPR Technology in with a... cular mechanisms: CRISPR: Gene editing and beyond | Nature Video

Pre-Lab | CRISPR-Cas9 vs CRISPR-Prime Editing

The CRISPR prime editing is an advanced gene-editing te...iqu... builds upon the original CRISPR-Cas9 system, offering more precise and ve...e edit... capabilities. While both systems utilize the Cas protein and a guide RNA, prime edit...n u... modified Cas9 protein called **Cas9 nickase (Cas9n)** and a specialized guide RNA known as **prime e...ing guide RNA (pegRNA)**.

A significant difference between CRISPR-Cas... and CRISP...ime editing is the way the DNA is cut. In the CRISPR-Cas9 system, the Cas9 pr...in c... double-strand break (DSB) in the target DNA, which can sometimes lead to u...tende... changes during the DNA repair process. In contrast, CRISPR prime editing uses the C...s9n t... make... single-strand nick in the DNA, reducing the likelihood of off-target effects rooting fro... non-h...mologous end joining process.

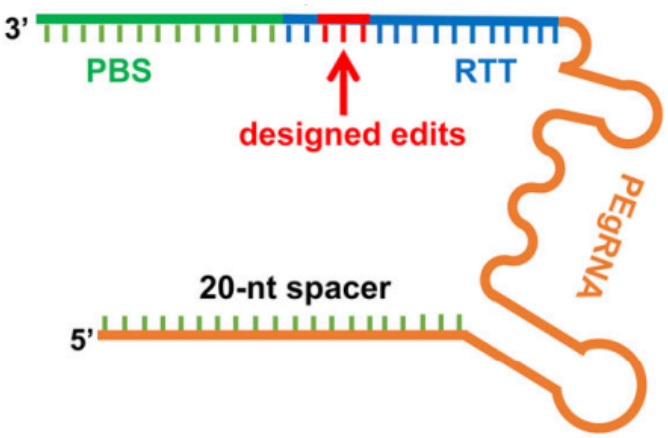
CRISPR prime editing also allow... for a...e...er variety of edits including precise insertions, deletions, and substitutions witho... the ne...d for a DNA repair template. The pegRNA in prime editing not only guides the Cas9n to the targe... but also **carries the template for the desired edit in its sequence**. To introduce the desired edit into the target DNA, the **M-MLV2 reverse transcriptase enzyme** is employed. T... e... me synthesizes a **complementary DNA (cDNA)** from the pegRNA, allowing the edited ...quence ... be copied directly into the target DNA during the reverse transcription process.

For refe...nce, the rev...se transcription process is a process by which RNA is converted back into DNA. This is ...ed to c...py the edited sequence from the pegRNA into the target DNA.

Feat...	CRISPR-Cas9	CRISPR Prime Editing
DNA ...tting	Double-Strand Break	Single-Strand Nick
Off-Target Effects	Higher Likelihood	Reduced Likelihood
Edit Types	Deletions, Insertions, and Substitutions (often require a repair template)	Precise Deletions, Insertions, and Substitutions (no repair template needed)
Enzyme Components	Cas9 Protein, Guide RNA, and repair template if needed	Cas9n, pegRNA, and -MLV2 reverse transcriptase

Table 1: Differences in the DNA cutting mechanism between CRISPR-Cas9 and CRISPR prime editing.

Pre-Lab | Designing Prime Editing Guide RNA (pegRNA) for CRISPR-PE System



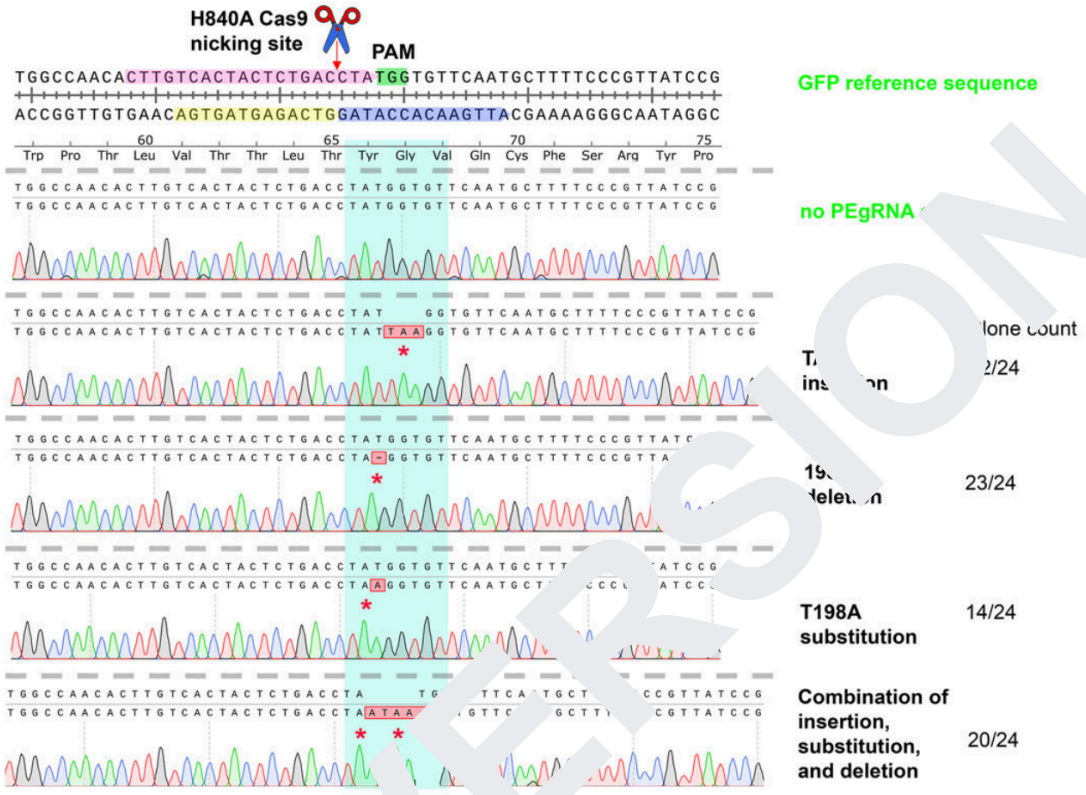
The steps to design a pegRNA for CRISPR prime editing includes:

1. **Target Sequence Selection**: Choose a target DNA sequence within your gene of interest, usually 20-nucleotide (nt) which should cover the region of the desired edits. Ensure that the target sequence has a **protospacer adjacent motif (PAM)** sequence such as NGG located downstream of the target site.

2. **Design Spacer**: Design a 20-nucleotide (nt) spacer sequence that is complementary to the target DNA sequence, excluding the PAM. The spacer sequence will guide the Cas9n-M-MLV2 complex to the target site.

3. **Primer Binding Site (PBS)**: Design a PBS sequence, usually around 13nt, that is complementary to the DNA strand with the PAM sequence, downstream of the target site. The PBS will bind to the nicked DNA strand and initiate reverse transcription. Note that the PBS sequence will perfect match part of the **Spacer** sequence.

4. **Reverse Transcription Template (RTT)**: Design the RTT containing the desired edit (insertion, deletion, substitution). The RTT should be complementary to the PBS-containing DNA strand but include the desired change(s). Note that part of the RTT sequence may not match that of the **Spacer** sequence.



pegRNA Design and Evaluation
Source: https://www.nature.com/articles/s41467-021-25541-3#Sec22

In the paper referenced for this lab, the authors designed a pegRNA for introducing premature stop codons into the GFP coding sequence. The target DNA sequence was chosen within the GFP coding sequence which has an appropriate **PAM** (NGG) downstream. The **spacer** (pink) was designed to be complementary to the target DNA sequence along with the **PBS** (yellow) and the **RTT** (blue) containing the desired edit with the TAA stop codon. This pegRNA guides the Cas9n-M-MLV2 complex to target site, allowing the introduction of a premature stop codon into the GFP coding sequence which leads to a loss of fluorescence.

Protocol | Part 1: Plasmids Preparation

Time Estimate: 90 minutes, 24 hour Incubation (the day before)

Plasmid Miniprep Materials

- Plasmid Miniprep Kit Protocol using Centrifugation (NEB #T1110)
- Buffer Preparation as recommended in Buffer Preparation Guidance

Plasmid Miniprep Protocol

1. **Pellet 1–5 ml bacterial culture (not to exceed 15 OD uni[illegible] centri[illegible]nation for 30 seconds in a 1.5mL microcentrifuge tube at maximum speed of [illegible]top [illegible]entrifuge (~14000 rpm). Discard the supernatant.** For a standard miniprep to p[illegible]e pla[illegible]mid for restriction digestion or PCR, we recommend 1.5 ml of culture, which is s[illegible]nt for most [illegible]pplications. Ensure cultures are not overgrown; 12–16 hours is usually ideal for [illegible]imal [illegible]owth.
   ***Note that if the culture were grown beyond [illegible]ho[illegible]n still proceed but should expect lower yield of plasmid DNA.

2. **Resuspend the pellet in 200 µl of M[illegible]n Buff[illegible] B1 (pink ●).** Vortex or pipet mix to ensure cells are completely resuspended. There should [illegible]visible clumps.

3. **Add 200 µl of Monarch Buff[illegible]2 [illegible]ue ●), gently invert the tube 5-6 times, and incubate at room temperature for 1 minute. Do not vortex.** The color should change to dark pink, and the solution should b[illegible]arent and viscous. Handle the sample gently to reduce the risk of shearing chromosomal [illegible]A, whi[illegible]can be co-purified as a contaminant. Avoid incubating longer than one minute to preve[illegible]rreversi[illegible] plasmid denaturation.

4. **Add [illegible] µl of Monar[illegible] Buffer B3 (yellow ●), and gently invert the tube until neutralized. Do not vortex.** T[illegible]color [illegible]ould be uniformly yellow and a precipitate will form. Incubate for 2 minutes. Gentle [illegible]rm [illegible]g will ensure complete neutralization without shearing chromosomal DNA.

5. **Centrifu[illegible]e the lysate for 2–5 minutes at maximum speed.** The pellet should be compact; spin [illegible]nger [illegible]needed. Spin time should not be less than 2 minutes. For culture volumes > 1 ml, we [illegible]mmend a longer spin (~5 minutes) to ensure efficient RNA removal by RNase A and a more compact pellet, which will lower the risk of clogging the column.

6. **Carefully transfer the supernatant to the Monarch Spin Column S2D and centrifuge for 1 minute. Discard the flow-through.**

7. **Re-insert the Monarch Spin Column S2D in the Monarch Spin Collection Tube and add 200 µl of Monarch Buffer BZ (wash 1). Centrifuge for 1 minute.** Discarding the flow-through is optional. This is a high-salt wash step that helps remove any residual RNA, protein, and other contaminants. Incubate for 5 minutes after adding Monarch Buffer BZ and before centrifugation if the plasmid will be used for transfection.

8. **Wash by adding 400 µl of Monarch Buffer WZ (wash 2) and centrifuge for 1 minute at maximum**

speed.

9. **Transfer the column to a clean 1.5 ml microfuge tube.** Use care to ensure that the tip of the column does not touch the
flow-through. If in doubt, re-spin for 1 minute.

10. **Add ≥ 30 µl of Monarch Buffer EY to the center of the matrix. Wait for 1 mi[illegible] then [illegible]n for 1 minute at maximum speed to elute DNA.** Nuclease-free water (pH 7– 8.5) can a[illegible]be u[illegible]elute the DNA. Yield may slightly increase if a larger volume of Monarch Buffer E[illegible]ed, [illegible] the DNA will be less concentrated. For larger size plasmids (≥ 15 kb), incubate the c[illegible]mn wi[illegible]n el[illegible]on buffer at room temperature for 5 minutes to maximize the yield. Alternatively, th[illegible]elution bu[illegible]r can be heated to 50°C before use.

Protocol | Part 2: Gibson Assembly

Time Estimate: 15 minute setup, 20 min wait.

Materials

- pegRNA Backbone
- Purified fragment 1
- Purified fragment 2
- Gibson Assembly Master Mix
- PCR tube
- UltraPure Water

Equipment

- Heat Block
- P20 pipette and 10uL tips

Protocol

1. Gibson Assembly - Paper pegRNA (from supplementary tabe 2):
    a. Setup the following reaction on ice. **Add the enzyme (Gibson Mix) last.** Notice the total volume is 10uL this time.

Reagent	Stock conc./amount	Desired conc./amount	Volume
pegRNA Backbone	40ng/uL	100ng	2.5
Purified fragment 1	???	13ng	
Gibson Assembly Master Mix	2X	1X	5uL
Nuclease-free water	n/a	n/a	Fill up to 10uL (if needed)
Total			10uL

2. Gibson Assembly - Custom pegRNA:
    a. Setup the following reaction on ice. Add the enzyme (Gibson Mix) last. Notice the total volume is 10uL this time.

Reagent	**Stock conc./amount**	**Desired conc./amount**	**Volume**
pegRNA Backbone	40ng/uL	100ng	2.5
Purified fragment 2	???	13ng	
Gibson Assembly Master Mix	2X	1X	
Nuclease-free water	n/a	n/a	Fill up to 10uL (if needed)
Total			10uL

3. Incubate in **50C** for **1 hour**
4. Store in 4C until the next lab session

Protocol | Part 3: Transformation into *E. coli*

Time

- 2 Hours working time and overnight incubation

Materials

- pCDF-GFP Plasmid
- pCRISPR-PE Plasmid
- Gibson Assembly x2
- NEB 5-alpha Competent E.coli
- SOC growth medium
- Two empty and labeled Microcentrifuge 1.5mL tubes
- LB agar plates with all appropriate antibiotics (better m ke t be ore as it takes time to autoclave and dry the plate)
    - Spectinomycin (Antibiotic) — 100 mg/m sto (10 n water
    - Ampicillin (Antibiotic) — 100 mg/mL ck ( ) in water
    - Chloramphenicol (Antibiotic) — 2 mg/m tock (1000x) in Ethanol
- 15ml Falcon tube for liquid waste dis osal

Equipment

- Ice bucket
- Waterbath set to 42C
- P1000 pipette with 1000uL tips
- P20 pipette with uL

Protocol

(based on http //www.addg .org/protocols/bacterial-transformation/)

1. T w mpe ells on ice (meaning: take a tube of cells out of the -80C freezer and put it directly ce, and wait until it becomes liquid). Takes 20 minutes.

2. Ta microcentrifuge tubes and label one as CRISPR-Paper and another as CRISPR-Custom with an additional identifiable marker for your team.

3. Transfer 5uL of all three plasmid DNA into your labeled tubes. Put the tube on ice.

4. Transfer 25uL of competent cells to each tube. Do NOT vortex. Mix GENTLY by flicking the bottom of the tube with your finger a few times.

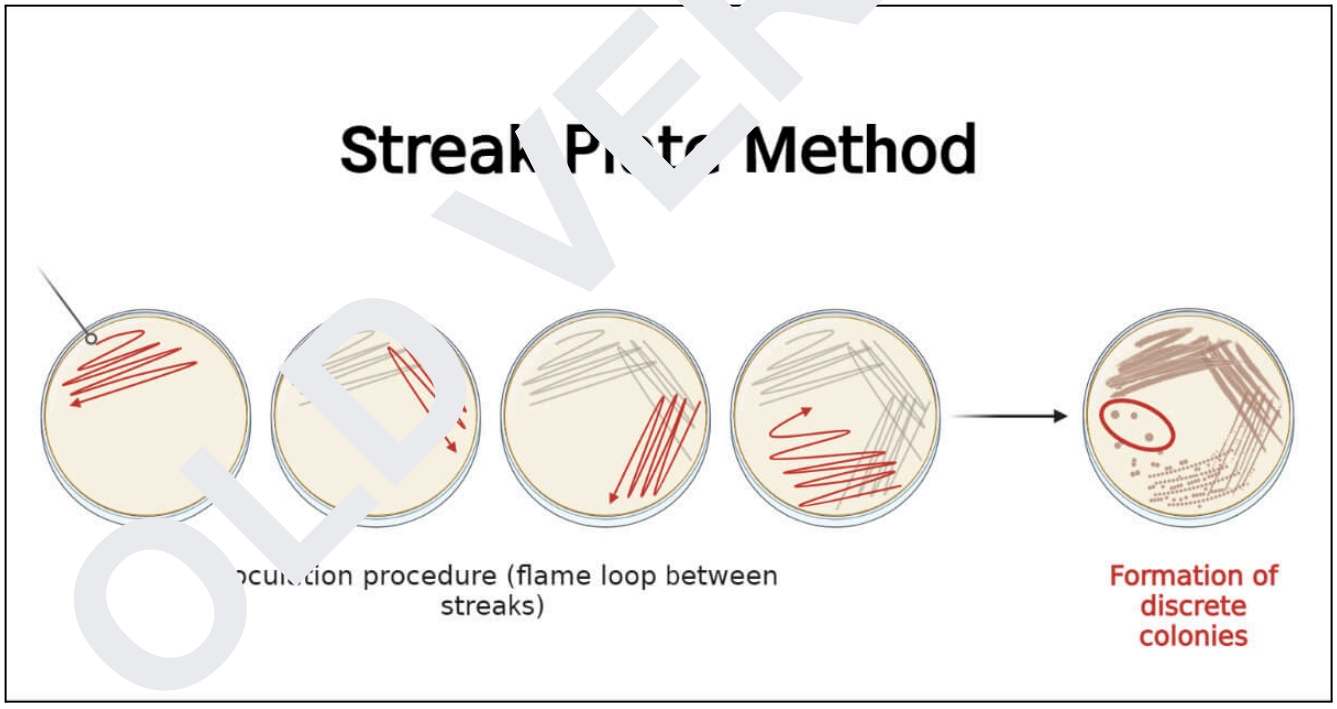
5. Incubate the tubes on ice for 15 minutes.

6. Take your ice bucket to the heat bath. Set up a timer for 45 seconds. With your hands holding both tubes, submerge the tubes in the 42C heat bath such that half of the tube is inside the hot water. Keep the tubes in 42C for exactly **45 seconds**, and transfer the tubes back to the ice for 2 minutes.

7. Add 250uL of SOC media to each of the tubes, and grow in a (shaking) incu[illegible] **minutes.**

8. Take 2 plates and label them:
    a. CRISPR-Paper
    b. CRISPR-Custom

9. Take two plates, transfer 20uL from each tube to its ap[illegible]ate, and use a sterile loop to drag through streak #1. Spread bacteria over [illegible]ond s[illegible] to create streak #2 and repeat to create a third section #3. See refer[illegible]ce pi[illegible]ure below.



Source: https://microbenotes.com/streak-plate-method-principle-methods-significance-limitations/

10. Incubate the plates at 37 C for 24 hours. Make sure you place the plates with the agar facing up. The reason we should face the agar up is because of condensation that might occur through overnight incubation. When water condensates drop onto the agar, they will create a smear rather than single colonies.

11. For all of the extra liquid you have that contains bacteria - transfer to the liquid waste disposal tube. Add bleach to kill all bacteria **(this how we always disposed of anything that has live bacteria in it for biosafety reason)**

Protocol | Part 4: Culturing and Analysis

Plate Reader Analysis

Time

- Setup: 1 Hour

LAB WILL BE AT BUILDING 26-033. : https://biomakers.mit.e[illegible]/ab[illegible]/hr[illegible]ms/

Conditions Present for pPegRNA

- 1T for control, etc
- New design TBD

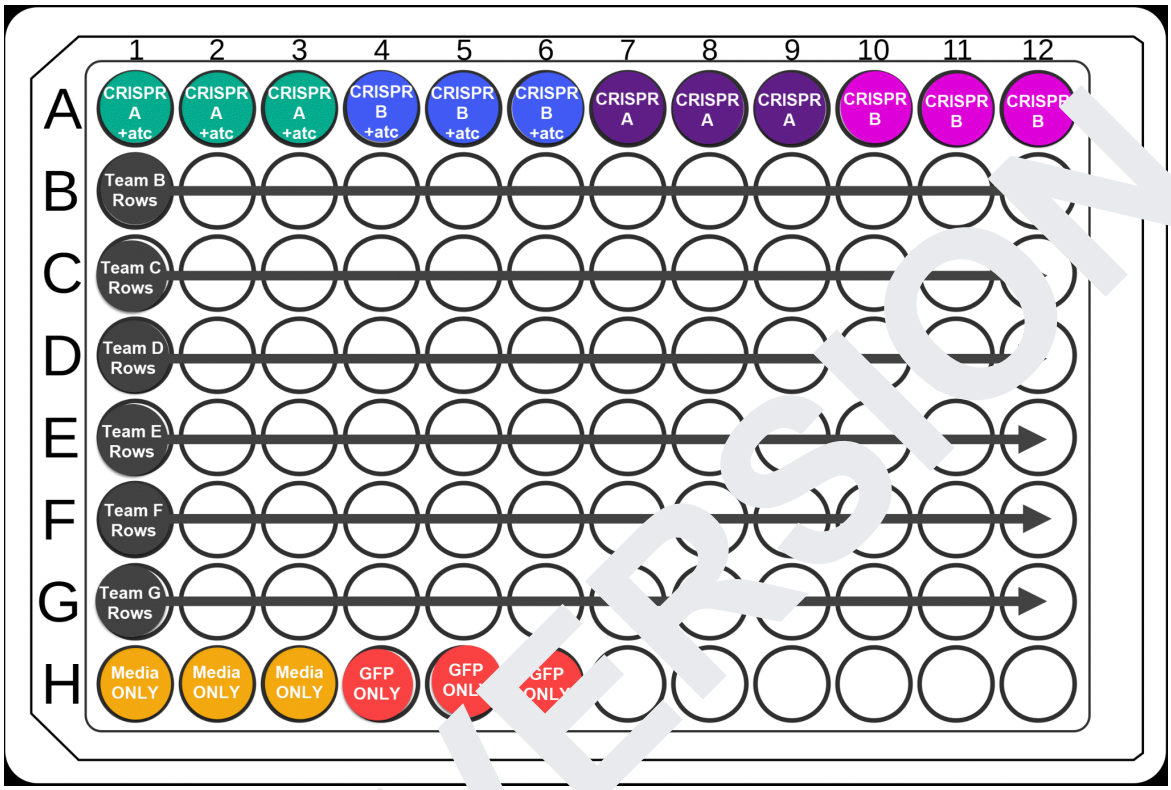
Materials

- Transformed E.coli colonies from [illegible]art I
- LB Media with appropriate antibiot[illegible]s
    - Streptomycin (Antibi[illegible]-1[illegible] mg/mL (1000x) in water
    - Ampicillin (Antibiotic) —100[illegible] mL (1000x) in water
    - Chloramph[illegible] (Antibiotic) —25 mg/mL (1000x) in Ethanol
- Anhydrotetracy[illegible]ne (aTc, [illegible] 200 ug/mL (1000x) in DMSO
- 96-well plate wit[illegible]lear bo[illegible]om and lid
- Microcentrifuge tub[illegible]

Equipme[illegible]

- [illegible]late R[illegible]der
- [illegible]1000 pi[illegible]tte with 1000uL tips
- [illegible]ette

Protocol



1. Label four culture tubes wi[illegible] fol[illegible]ving labels:
    a. CRISPR-Paper +atc
    b. CRISPR Pa[illegible]
    c. CRISPR [illegible]ustom[illegible]tc
    d. CRISPR[illegible]stom

2. Add 5m[illegible]f LB media [illegible]with all three antibiotics (Spectinomycin, Ampicillin, Chloramphenicol) to each of [illegible] cul[illegible]e tubes.

3. [illegible]For each [illegible]lture tube, scrape a colony using a sterile pipette tip from the corresponding plate [illegible]ther C[illegible]SPR A or CRISPR B) and place them in the culture tube.

4. Incubate for 30 minutes at 37C.

5. Add **.5uL** of 2mg/mL **anhydrotetracycline (atc)** to the culture **tubes labeled with +atc** to achieve a final concentration of 200ng/mL. Vortex to incorporate.

6. Fill wells 1-3 in your team's row with 200uL per well of the **CRISPR Paper +atc** liquid culture.

7. Fill wells 4-6 in your team's row with 200uL per well of the **CRISPR Custom +atc** liquid culture.

8. Fill wells 7-9 in your team's row with 200uL per well of the **CRISPR Paper** liquid culture.

9. Fill wells 10-12 in your team's row with 200uL per well of the **CRISPR Custom** liquid culture. Note that wells 7-12 do not contain atc. These will act as a control to test the induction of the CRISPR plasmid. Also note that row H wil contain additional controls including "media only" as a negative control and an e.coli culture transformed with only the pCDF-GFP plasmid as a positive control.

10. Cover the 96-well plate with a lid and incubate in a plate reader at 37°C with shaking. Program the plate reader to measure **OD630** and GFP (**excitation: 488nm, emission wavelengths: 509nm**) every 20 minutes for 12-24 hours. Note we are using OD630 to avoid potential interference from the GFP fluorescence.

11. The TAs will send you the data after incubation. You will need to plot the normalized GFP fluorescence (GFP/OD630) against time for each well. Normalize the fluorescence values with the corresponding OD630 values and compare them against the controls to determine the efficiency of the CRISPR prime editing system.

Suggest using GFP/OD630 as Y-axis and time as X-axis for plotting. You may notice that the value at the earlier time might be conflicting since the denominator might be close to zero.