For educational use only.

CRISPR IN A BOX™

Educational Kits

Instruction Manual

Dear Instructor

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) technology has been used globally for human gene editing, allowing for manipulations of the genome to be possible. This has given researchers the opportunity to utilize an innovative tool to repair point mutations, mutagenetic deletions, and disabled genes, which contribute to the development of threatening diseases.

CRISPR was originally identified as an adaptive tool used in bacterial immune defense. It was discovered that during viral infection, bacterial defenses chopped up the invading DNA and integrated it into the host genome. Bacteria could then generate CRISPR components and form RNA-Cas complexes so that if viral DNA is re-introduced, the bacteria could target this invading DNA for destruction.

The CRISPR in a Box™ Educational Kits utilize this revolutionizing CRISPR-Cas technology. The model system provided uses CRISPR-Cas12a ribonucleoprotein (RNP) *in vitro* to modify a segment within the lacZ gene using a single-stranded donor DNA template and a mammalian cell-free extract. The single-stranded donor DNA template contains flanking arms of homology to the target site and directs the integration of a *Not*I restriction enzyme site. The products of this reaction are transformed into *Escherichia coli* (*E. coli*) bacteria and a visual, phenotypic readout of a blue-to-white color change is seen. Additional analysis including restriction enzyme digestion or sequencing can be can be used to confirm presence of the integrated *Not*I site.

The main advantage of using CRISPR in a Box™ Educational Kits, which utilizes the *in vitro* system, is the ability to understand the basics of different repair pathways and the importance of the revolutionizing gene editing tool, CRISPR/Cas. By using this kit, your students will be able to understand where CRISPR/Cas comes from, and the difference between HDR and NHEJ repair pathways which can occur when DNA is damaged.

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Safety Guidelines

Follow basic laboratory safety rules and wear recommended personal protective equipment including protective gloves, protective clothing, and eye/face protection. Always wash hands and wrists thoroughly with soap and water before leaving the laboratory, even if gloves were worn during the work day. In case of an accidental splash and solution gets into the eyes, rinse with water for 15 minutes.

Several components in this kit may cause skin irritation. In case of direct contact with the skin, wash with abundant water for 10 minutes. Carbenicillin is an antibiotic that may cause allergic reactions and those with allergy to antibiotics should consult with their physician before handling the materials included in the kit.

The *E. coli* DH5-Alpha strain (DH5α) is an engineered non-pathogenic strain of bacteria used for routine subcloning procedures. However, always use standard safety microbiological practices when working with the kit. Before disposal, treat bacterial culture, plates, and any other materials that contacted bacteria with bleach (10% [v/v] final concentration) for at least 30 minutes. Consult the local institutional safety officer for specific handling and disposal procedures.

KIT COMPONENTS

Note: Each kit contains reagents for 5 workstations (4 experimental setups and 1 negative control setup) for a total of 10 reactions.

Reagents Included

■ Starter Kit ■ Complete Gene Editing Laboratory Kit

Step	Reagent	Quantity	Storage	Catalog #	Starter Kit	Complete Gene Editing Laboratory Kit
In Vitro Gene Editing	10X 3.1 Buffer	25 µL	–20°C	KGA-0100S		✓
	Carbenicillin	600 µL	–20°C	KGA-0100T		✓
	Cas12a Nuclease	2 µL	–20°C	KGA-0100A	✓	✓
	Cell-Free Extract	50 µL	–20°C	KGA-0100C	✓	✓
	crRNA1364	10 µL	–20°C	KGA0100B	✓	✓
	HDR-NS Oligonucleotide	10 µL	–20°C	KGA-0100D	✓	✓
	IPTG	700 µL	–20°C	KGA-0100K		✓
	pUC19 Plasmid	50 µL	–20°C	KGA-0100V		✓
	Ligase	12 µL	–20°C	KGA-0100H		✓
	X-gal	700 µL	–20°C	KGA-0100L		✓
	10X In Vitro Reaction Buffer	Lyophilized	2–8°C	KGA-0100N		✓
	Cas Buffer	100 µL	2–8°C	KGA-0100E		✓
	RNA Buffer	100 µL	2–8°C	KGA-0100M		✓
	C&C Binding Buffer	1.3 mL	RT	KGA-0100Y		✓
	C&C Wash Buffer	11 mL	RT	KGA-0100Z		✓
	LB Agar Powder	18 g	RT	KGA-0100U		✓
	UltraPure Water	2 mL	RT	KGA-0100J		✓
Miniprep	Miniprep P1	6.3 mL	2–8°C	KGA-0100EE		✓
	LB Broth Capsules	2 Capsules (80 mL)	RT	KGA-0100CC		✓
	Miniprep Binding Buffer	6.9 mL	RT	KGA-0100HH		✓
	Miniprep P2	6.3 mL	RT	KGA-0100FF		✓
	Miniprep P3	6.3 mL	RT	KGA-0100GG		✓
	Miniprep Wash 1	19.8 mL	RT	KGA-0100JJ		✓
	Miniprep Wash 2	24 mL	RT	KGA-0100KK		✓
Competent Cells Preparation	2X Competent Buffer	83 µL	2–8°C	KGA-0100PP		✓
	2X Wash Buffer	83 µL	2–8°C	KGA-0100P		✓
	DH5α *E. coli*	Lyophilized	2–8°C	KGA-0100F		✓
	Dilution Buffer	1.7 mL	2–8°C	KGA-0100G		✓
	ZymoBroth™	18 mL	RT	KGA-0100X		✓
Double Restriction Digest	10X Digestion Buffer	52 µL	–20°C	KGA-0100AA		✓
	*Not*I Restriction Enzyme	26 µL	–20°C	KGA-0100DD		✓
	*Xmn*I Restriction Enzyme	26 µL	–20°C	KGA-0100YY		✓

Gel Electrophoresis	Diamond Ready-to-use DNA Ladder (0.1–10 Kbp)	11 µL	2–8˚C	KGA-0100Q		✓
	50X TAE Buffer	22.5 mL	RT	KGA-0100R		✓
	6X Sample Loading Dye	80 µL	RT	KGA-0100BB		✓
	Agarose	1.1 g	RT	KGA-0100NN		✓
	SYBR™ Safe DNA Gel Stain	11 µL	RT	KGA-0100VV		✓
Other Consumables	0.2 mL Tubes	50	RT	KGA-0100TT		✓
	1.5 mL Tubes	190	RT	KGA-0100RR		✓
	C&C Collection Tubes	20	RT	KGA-0100WW		✓
	C&C Spin Columns	20	RT	KGA-0100ZZ		✓
	Inoculating Loops	20	RT	KGA-0100XX		✓
	Inoculation Spreaders	20	RT	KGA-0100SS		✓
	Miniprep Collection Tubes	20	RT	KGA-0100MM		✓
	Miniprep Spin Columns	20	RT	KGA-0100LL		✓
	Petri Dishes	20	RT	KGA-0100QQ		✓
	Round Bottom Tubes	22	RT	KGA-0100UU		✓

Materials Required but Not Included

Reagents	Quantity
Adjustable-volume Micropipettes: 2–20, 20–200, and 200–1,000 µL	≥ 5 each
Autoclavable Bottles with Caps: 250 mL and 1 L	1 each
Autoclave or Microwave Oven	1
Benchtop Centrifuge	≥ 1
Blue Light or UV Transilluminator	1
Deionized Water	2 L
Erlenmeyer Flasks: 125 mL and 250 mL	1 each
Gloves: Small, Medium, and Large	≥ 1 box each
Graduated Cylinders (100 mL, 500 mL, and 1 L)	1 each
Horizontal Gel Electrophoresis Chamber with Gel Casting Tray and Comb	≥ 1
Micropipette Tips: 20, 200, and 1,000 µL	≥ 5 boxes each
Permanent Marking Pens	≥ 5
Power Supply	≥ 1
Shaking Incubator	1
Stationary Incubator	1
Spectrophotometer and Associated Materials	1
Temperature-controlled Dry Bath or Water Bath	1
Tube Racks	≥ 5
Wet Ice and Containers (e.g., ice bucket)	≥ 5

BACKGROUND INFORMATION

CRISPR/Cas System

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) technology has been used globally for human gene editing, allowing for manipulations of the genome to be possible. This has given researchers the opportunity to utilize an innovative tool to repair point mutations, mutagenetic deletions, and disabled genes, which contribute to the development of threatening diseases.

CRISPR was originally identified as an adaptive tool used in bacterial immune defense. It was discovered that during viral infection, bacterial defenses chopped up the invading DNA and integrated it into the host genome. Bacteria could then generate CRISPR components and form RNA-Cas complexes so that if viral DNA is re-introduced, the bacteria could target this invading DNA for destruction. This is often known as adaptive immunity.

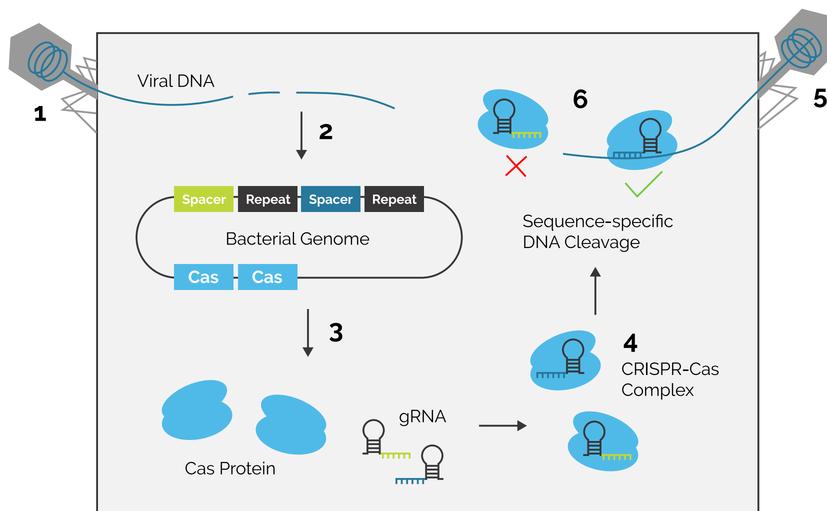


Figure 1: Adaptive Immunity Process in Bacteria.
If a bacterial cell is infected by a virus (1), the cell integrates the viral DNA into its genome (2). The bacteria then makes the CRISPR-Cas components (3), which form the gRNA-Cas complex (4). If the bacteria are reinfected by the same virus (5), the complexes cleave the viral DNA and reinfection is prevented (6).

Cas12a Nuclease

Cas12a formerly known as Cpf1, is a nuclease derived from the microorganism known as *Acidaminococcus sp.* Some of Cas12a's important features include the protospacer adjacent motif, commonly referred to as PAM, which is vital in finding potential places for the Cas12a nuclease to bind to target DNA. The PAM site is T rich and requires a 5' – TTTN – 3' composition where N is any of the four bases of DNA (A, T, G, and C).

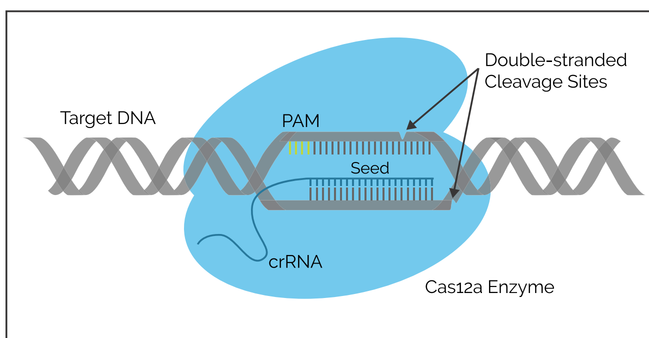
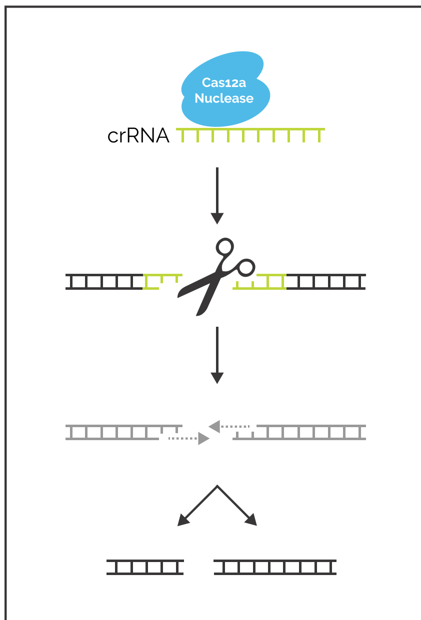


Figure 2: Cas12a ribonucleoprotein and key features. Cas12a is bound to the target DNA in conjunction with the crRNA. This entire structure encompasses the ribonucleoprotein (RNP). The PAM site is represented by the four green lines on the top left of the target DNA within the RNP. The double-stranded staggered cleavage site is represented by two triangles cut out of the target DNA, one on the top and one on the bottom of the target DNA on the right side of the image.

The next key feature is the complexation of Cas12a nuclease and CRISPR RNA (crRNA) that forms a ribonucleoprotein (RNP). The crRNA helps the RNP bind to the target DNA that is going to be edited. The crRNA is 21 base pairs in length and follows directly after the PAM site. Once the RNP recognizes the target site, Cas12a can then cleave the target DNA with a double-stranded staggered cut, generating a 5-base pair, 5' overhang.

Gene Editing Pathways

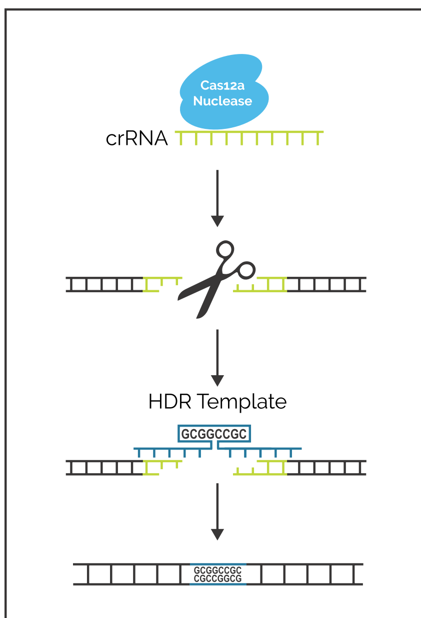
Non-Homologous End Joining Pathway



Non-Homologous End Joining (NHEJ) is a damage repair pathway that can occur when DNA is damaged or intentionally cleaved by a CRISPR-Cas12a RNP. When cleavage of the DNA happens, the ends of the exposed DNA can be degraded causing a few of the nucleotides on one or both sides to be lost or added. The DNA will repair itself quickly, causing potential mistakes to be made. The resulting DNA can be missing a few base pairs or contain extra base pairs causing the DNA to be shorter or longer. In gene editing, these changes can be referred to as a gene knock-out.

Figure 3: Non-homologous End Joining Pathway. The Cas12a nuclease and crRNA, which make up the RNP, are shown at the top. The Cas12a RNP (represented by the scissors) cuts the DNA and exposes cut ends of the DNA. The ends of the DNA are then repaired by cellular mechanisms shown at the bottom of the figure.

Homology Directed Repair Pathway



Homology Directed Repair (HDR) is a repair pathway that is activated when DNA has a double-stranded break and is provided with a single-stranded or double-stranded template. The template must properly bind and align to the DNA. The result of this could be a long or short piece of DNA that is used as a template for DNA repair machinery to fill in the gap created by the CRISPR-Cas12a RNP.

Figure 4: Homology Directed Repair Pathway. The Cas12a nuclease and crRNA, which make up the RNP, are shown at the top. The RNP (represented by the scissors) cleaves the DNA resulting in a staggered double-stranded break. The homology directed repair template is shown with homology arms on either side of the break site properly binding to the target DNA. The site shown in the middle is then integrated into the DNA shown in the final image.

pUC19 Plasmid and the lacZ Gene

This kit will edit the lacZ gene in the pUC19 plasmid (Fig. 5). After the gene editing reaction is complete, the plasmid is transformed into *E. coli* for the visual, phenotypic readout. The lacZ gene is part of the inducible lac operon found in bacteria and is turned on in the presence of lactose.

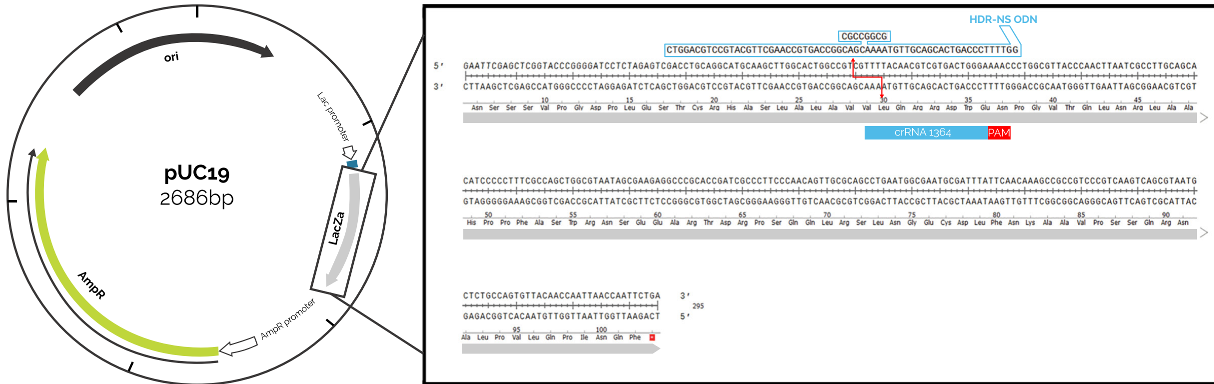


Figure 5: LacZ gene and important features. The pUC19 plasmid is shown on the left with a black box highlighting the lacZ gene. The right side of the image shows part of the of the lacZ gene sequence which has been amplified to illustrate the binding site of the 1364 crRNA, HDR-NS oligonucleotide template (in blue) and the staggered cut site created by the RNP illustrated by the red arrow.

Lactose is difficult to digest so the operon's function is to break lactose down into simpler sugars such as glucose and galactose. The lacZ gene produces an enzyme called β-galactosidase. This enzyme can be detected with the use of a chemical known as X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). If the lacZ gene is working correctly, β-galactosidase will be produced and cleave X-gal. When X-gal is cleaved, it produces a bright blue color that changes the colony color from white to blue.

There is one additional chemical that must be added to the growth medium to ensure that the lac operon will turn on and produce β-galactosidase. This chemical is known as IPTG (Isopropyl β-D-1-thiogalactopyranoside) and serves to turn on the lac operon without lactose. As shown in Figure 6, when IPTG is added, the repressor protein that normally prevents the genes from being transcribed, will bind IPTG and come off of the operator. This enables the lacZ gene to be transcribed and make -galactosidase.

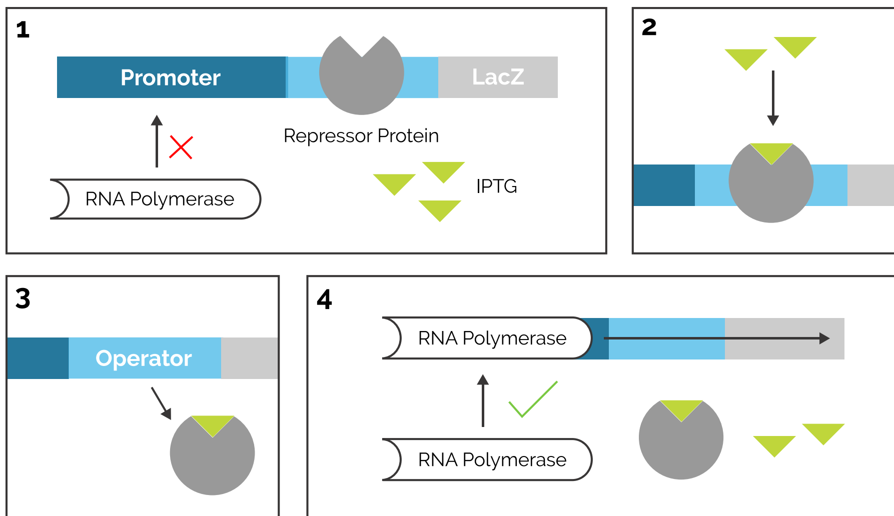


Figure 6: IPTG and the Lac operon. Transcription of the lac operon is achieved through the use of IPTG. Panel 1 shows the repressor protein bound to the operator so that RNA Polymerase cannot bind. Panel 2 represents IPTG binding to repressor. Panel 3 represents the repressor being released from the operator. Panel 4 shows RNA polymerase bound to the operon so transcription can proceed.

Common Acronyms

- CFE: Cell-Free Extract
- CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats
- crRNA: CRISPR RNA
- DNA: Deoxyribonucleic Acid
- HDR: Homology Directed Repair
- INDELS: Insertions and deletions
- NHEJ: Non-Homologous End Joining
- ODN: Oligonucleotide
- PAM: Protospacer Adjacent Motif
- RNA: Ribonucleic Acid
- RNP: Ribonucleoprotein

EXPERIMENTAL OVERVIEW

In Vitro Gene Editing Reaction

The *in vitro* gene editing reaction is designed to walk students through an experiment that has been used in a gene editing laboratory and has been used as the foundation for multiple research publications (see References). The overall experiment is broken into eight big concepts. Students will generate an RNP complex and then cut plasmid DNA at the lacZ gene. The linearized plasmid will be cleaned up and then repaired using a donor repair template and cell-free extract. The recircularized plasmid will be cleaned again and then transformed into competent *E. coli* cells for a blue/white colony readout. Plasmids that have been gene edited will present as white because the lacZ gene has been disrupted. The repair template encodes a *Not*I restriction enzyme site to be used for a genotypic readout in addition to the phenotypical colony readout.

Plasmid DNA Miniprep

Blue and white *E. coli* colonies will be grown overnight to perform a miniprep that will obtain the gene edited plasmid. The recovered plasmid can be used for different downstream analyses. This kit provides the materials for a restriction enzyme digestion based on the plasmid DNA. DNA sequencing may also be performed using the recovered plasmid DNA to illustrate the different types of outcomes that a gene editing reaction can generate.

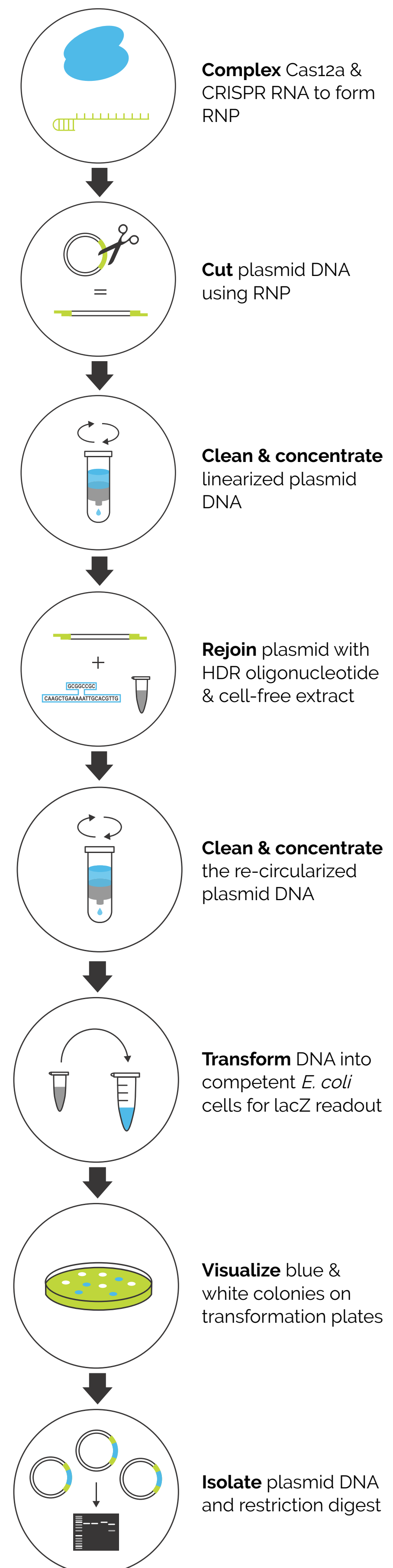
Restriction Enzyme Analysis

A restriction enzyme digestion is a visual confirmation of the presence of a gene edited colony containing a *Not*I site (GCGGCCGC). This will allow the students to see the difference between non-gene edited colonies (blue) and edited colonies (white).

To aid the visualization of gene editing during the genotyping step, a second restriction enzyme, *Xmn*I in addition to *Not*I, is included in the kit. Non-gene edited colonies will produce a single band, while edited colonies produce double bands during gel electrophoresis after the restriction enzyme digestion.

It is important to note that some white colonies may not show a double band on a gel electrophoresis. This indicates other gene editing events have possibly occurred such as a deletion or an insertion which can be assessed by sanger sequencing.

For further explanation, please see the diagram of non-homologous end joining explanation (NHEJ) in Figure 3.



PREPARATION CHECKLIST

Up to 1 Week in Advance

- [ ] Prepare medium for bacterial growth (pg. 12)

Day Before *In Vitro* Reaction

- [ ] Start overnight culture for competent cells (pg. 12)

Day of *In Vitro* Reaction

- [ ] Preparation of competent cells (pg. 12)
- [ ] Setup of experimental reagents (pg. 13–14)
- [ ] Preparation of student workstations (pg. 14)
- [ ] *In vitro* reaction protocol (for students) (pg. 15–16)

Day Before Miniprep Reaction

- [ ] Start overnight cultures of blue and white colonies (pg. 17)

Day of Miniprep Reaction

- [ ] Preparation of student workstations (pg. 17)
- [ ] Miniprep protocol (for students) (pg. 18)

Day of Restriction Digestion Reaction

- [ ] Preparation of master mix (pg. 19)
- [ ] Preparation of agarose gel (pg. 19)
- [ ] Digestion reaction protocol (for students) (pg. 20)

IN VITRO REACTION PREPARATION

Up to 1 Week in Advance

1. Prepare LB Agar plates with X-gal, IPTG, and Carbenicillin
   a. To a 1 L glass bottle, add
      - 500 mL deionized water
      - 18 g LB Agar Powder (entire bottle)
   b. Autoclave media at 121˚C for 30 minutes, 15 psi (see note below)
   c. Cool to approximately 60˚C
   d. Add:
      - 500 µL of X-gal
      - 500 µL of IPTG
      - 500 µL of Carbenicillin
   e. Mix thoroughly by slowly swirling
   f. Pour 20–25 mL of medium per 100 mm plate
   g. Allow to cool and store at 4˚C until needed

2. Prepare LB Broth
   a. To a 250 mL glass bottle, add:
      - 80 mL deionized water
      - 2 x capsules of LB broth
   b. Autoclave media at 121˚C for 30 minutes, 15 psi (see note below)
   c. Allow to cool while swirling occasionally and store at 4˚C until needed

Note: Although autoclaving is preferred for agar and broth preparation, a microwave can also be used. Microwave the media for 60–90 seconds to bring to a boil and dissolve ingredients. Boil vigorously for 5 seconds. Microwaving too long will boil the solution over. The solution should appear clear with no visible media granules. If not, continue microwaving in 10–15 second bursts until all components are dissolved.

Day before *In Vitro* Reaction

1. Start overnight DH5α *E. coli* culture 16–20 hours before preparing competent cells
   a. Reconstitute lyophilized DH5α *E. coli* by adding 200 µL of UltraPure Water
   b. Add 1 mL of LB broth (no antibiotic) to a round bottom tube
   c. Add 20 µL of reconstituted DH5α *E. coli* to the tube
   d. Incubate culture at 37˚C with shaking (≥200 rpm) for 16–20 hours

Day of *In Vitro* Reaction

1. Prepare competent cells
   a. Prepare a 125 mL Erlenmeyer flask by adding 16 mL of ZymoBroth™
   b. To the broth, add 160 µL of overnight culture grown from the day before
   c. Swirl thoroughly to mix
   d. Grow the culture at 37˚C with shaking (≥200 rpm) until an Optical Density (OD) of 0.4–0.5 has been reached (approximately 3–5 hours) and record as follows:
      - Blank a spectrophotometer set to 600 nm with ZymoBroth™
      - Record the initial OD of the 16 mL culture with bacterial overnight added
   e. While the cells are growing, pre-chill 2X Wash Buffer, 2X Competent Buffer, and Dilution Buffer on ice prior to using in the preparation
   f. Dilute the 2X Wash Buffer and 2X Competent Buffer to 1X concentration using

the Dilution Buffer as written below:

- To a 1.5 mL tube, add 600 µL of Dilution Buffer to 600 µL of 2X Wash Buffer
- To a 1.5 mL tube, add 600 µL of Dilution Buffer to 600 µL of 2X Competent Buffer

g. After optimal density has been reached, incubate the culture on ice for at least 10 minutes
h. Swirl culture to mix and transfer 12 mL to a centrifuge tube
i. Centrifuge culture at 3,500 rpm at 4˚C for 20 minutes. Discard supernatant
j. Gently resuspend the cells by pipetting up and down in 1.2 mL ice-cold 1X Wash Buffer
k. Repeat centrifugation. Completely remove supernatant.
l. While centrifuging, set up (10) 1.5 mL tubes on ice for the cells to be aliquoted into
m. Gently resuspend the cells in 1.2 mL ice-cold 1X Competent Buffer by pipetting up and down
n. Aliquot 100 µL of cells per 5 mL tube

If using a room temperature microcentrifuge, substitute the following steps:

h. Aliquot 1 mL of culture into (12) pre-chilled 1.5 mL tubes
i. Centrifuge culture at 6,000 x g for 5 minutes
j. Gently resuspend each tube by pipetting up and down in 100 µL ice-cold 1X Wash Buffer
k. Repeat centrifugation. Completely remove supernatant
l. Disregard this step
m. Gently resuspend each tube in 100 µL ice-cold 1X Competent buffer by pipetting up and down
n. Disregard this step

o. Incubate tubes on ice for 30 minutes
p. Cells are now ready to be transformed with the *in vitro* reaction following the "DH5α Competent *E. coli* Transformation" protocol on page 16

Note: If unable to prepare competent cells on the same day as the *in vitro* reaction with the students, the prepared competent cells may be stored at 4˚C for up to 24 hours; however, there will be a decrease in transformation efficiency.

For the following steps (2–6), store reagents on ice during & after preparation.

2. Prepare Cas12a Nuclease
    a. Centrifuge tube of Cas12a protein at maximum speed for 30 seconds
    b. Add 68.2 µL of Cas Buffer directly to the tube of Cas12a to make 2 µM
    c. Mix by pipetting up and down 5 times
    d. Place a check mark on top of the tube to indicate complete preparation

3. Prepare crRNA1364
    a. Centrifuge tube of crRNA1364 at maximum speed for 30 seconds
    b. Add 48 µL of RNA Buffer directly to the tube of crRNA1364 to make 2 µM
    c. Mix by pipetting up and down 5 times
    d. Place a check mark on top of the tube to indicate complete preparation

4. Prepare 10X *In Vitro* Reaction Buffer
    a. Add 100 µL of UltraPure Water to the tube of lyophilized 10X *In Vitro* Reaction Buffer

b. Mix by pipetting up and down 5–10 times until completely dissolved
c. Place a check mark on top of the tube to indicate complete preparation

5. Prepare HDR-NS Oligonucleotide (ODN)
    a. Centrifuge tube of HDR-NS ODN at maximum speed for 30 seconds
    b. Add 12 µL of UltraPure Water directly to the HDR-NS ODN tube to make 50 µM of HDR-NS ODN
    c. Mix by pipetting up and down 5 times
    d. Place a check mark on top of the tube to indicate complete preparation

6. Prepare Cell-Free Extract (CFE) + Ligase mixture
    a. Add 11 µL of Ligase directly to the tube of CFE
    b. Mix thoroughly by pipetting up and down 5 times
    c. Place a check mark on top of the tube to indicate complete preparation

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Student Workstation Setup

Note: There will be 5 student workstations. Each workstation aliquot will be enough for 2 reactions.

1. Prepare *In Vitro* Reaction workstations using the following tables:

Consumable	# per Workstation
0.2 mL Tubes	4
1.5 mL Tubes	4
C&C Collection Tubes	4
C&C Spin Columns	4
Inoculation Spreaders	4
Prepared Agar Plates	4

Note: Prepare reagents on ice or cold block. Workstation #5 (negative control) will NOT receive Cas12a Nuclease; instead, add UltraPure Water. To stay consistent with tube numbers, aliquot 11 µL into a 1.5 mL tube and label "Cas12a Negative".

Reagent	Volume per Workstation	# of 1.5 mL Tubes
10X 3.1 Buffer	4.4 µL	5
10X *In Vitro* Reaction Buffer	6.6 µL	5
C&C Binding Buffer	220 µL	5
Cas12 Nuclease (see note before preparation)	11 µL	4
CFE + Ligase	11 µL	5
crRNA1364	11 µL	5
HDR-NS ODN	4.4 µL	5
Plasmid DNA (250 ng/µL)	8.8 µL	5
UltraPure Water	150 µL	5

Note: C&C Wash Buffer is needed but should not be aliquoted to avoid evaporation.

IN VITRO REACTION PROTOCOL: HOMOLOGY DIRECTED REPAIR IN LACZ

Note: Ensure reaction mixture remains on ice to be kept cold during preparation. This can also be done using a mini tube cooler or freezer box.

Cas12a – crRNA Complex (RNP Complex)

1. To a 0.2mL tube, add:
   a. 5 µL of Cas12a protein
   b. 5 µL of crRNA1364
2. Mix gently by flicking the tube
3. Incubate at room temperature for 15 minutes

Cleavage Reaction

4. Prepare cleavage reaction by adding the components as shown below to the tube containing the RNP complex (10 µL)

Component	Preparation
UltraPure Water	4 µL
10X 3.1 Buffer	2 µL
Plasmid DNA (250 ng/µL)	4 µL
Final Volume	**20 µL**

5. Incubate at 37˚C for 15 minutes

DNA Clean & Concentrate I

6. To the cleavage reaction tube, add 40 µL C&C Binding Buffer
7. Pipet up and down to mix until homogenous
8. Transfer all solutions to a spin column placed in a C&C Collection Tube
9. Centrifuge for 1 minute at 10,000 x g, discard flow through
10. Add 200 µL C&C Wash Buffer to the column
11. Centrifuge for 1 minute at 10,000 x g, discard flow through
12. Repeat steps 10–11 for a second wash
13. Transfer the spin column to a new 1.5 mL microcentrifuge tube
14. Add 25 µL UltraPure Water directly to the filter
15. Incubate at room temperature for 1 minute
16. Centrifuge for 1 minute at 10,000 x g
17. This recovered DNA will be used in the following reaction

Note: The eluted DNA may be stored for up to 1 week at –20˚C.

Recircularization Reaction

18. To a new 0.2 mL tube, add reagents as shown below
    Note: Prepare on ice or cold block

Component	Preparation
Recovered DNA	20 µL
HDR-NS Oligonucleotide (ODN)	2 µL
10X *In Vitro* Reaction Buffer	3 µL
CFE + Ligase	5 µL
Final Volume	**30 µL**

19. Incubate at 37˚C for 15 minutes

DNA Clean & Concentrate II

*Refer to 'DNA Clean & Concentrate I' diagram on page 15.

20. To the recircularization reaction tube, add 60 µL C&C Binding Buffer
21. Pipet up and down to mix until homogenous
22. Transfer all solutions to a C&C Spin Column placed in a C&C Collection Tube
23. Centrifuge for 1 minute at 10,000 x g, discard flow through
24. Add 200 µL C&C Wash Buffer to the column
25. Centrifuge for 1 minute at 10,000 x g, discard flow through
26. Repeat steps 24–25 for a second wash
27. Transfer the spin column to a new 1.5 mL microcentrifuge tube
28. Add 25 µL UltraPure Water directly to the filter
29. Incubate at room temperature for 1 minute
30. Centrifuge for 1 minute at 10,000 x g

Note: This is the final edited DNA. It may be stored up to 1 month at –20˚C.

DH5 Competent *E. coli* Transformation

31. Prewarm the LB/Carb/X-gal/IPTG plates at 37˚C for about 30 minutes prior to starting the transformation to remove any excess moisture and cause less shock to the bacteria when plated
32. Flick the tube of prepared competent cells to mix prior to adding DNA
33. Add 5 µL of the final edited DNA (from step 30) to the cells and flick the tube to mix thoroughly
34. Incubate cells on ice for 5 minutes
35. Plate the cells using a bacterial spreader by adding 50 µL of cell to a plate. Perform this step twice so that the entire transformation reaction is plated onto 2 total plates
36. Incubate the plates, inverted, overnight at 37˚C
37. 16–20 hours after transformation, white and blue colonies will be visible
38. Store the reaction plates at 4˚C for up to 2 weeks

To increase transformation efficiency, follow the following steps after step 34:

a. Heat shock the cells at 42C for 30 seconds
b. Incubate on ice for 2 minutes
c. Add 900 µL SOC media
d. Incubate at 37˚C with shaking for 1 hour
e. Spin down culture and remove approximately 850 µL
f. Proceed to step 35

PLASMID MINIPREP PREPARATION

Day Before Miniprep

1. Add 80 µL of Carbenicillin to the 80 mL LB Broth. Shake to mix fully
2. For overnight cultures of bacterial colonies
   - Prepare 20 Round Bottom Tubes for the culture
     Note: Label 10 tubes "white" and 10 tubes "blue" and number 1–10 respectively to help students understand which colony color is added to the tube.
   - Add 2 mL of LB/Carbenicillin broth to each tube
   - Using a new inoculating loop for each colony, pick 1 white colony and inoculate a 2 mL LB/Carbenicillin broth by swirling it in the broth
   - Continue until all 10 colonies are picked and then start selecting blue colonies
     Note: Remember, only pick blue colonies from the experimental plates and NOT the negative control plates
   - Incubate the cultures at 37˚C with shaking (225 rpm) for 12–16 hours
   - The following day the cultures should be turbid and ready for the plasmid miniprep procedure
   - Store at 4˚C until ready to use

Note: If there are less than 10 colonies of either color, one colony may be split into 2–3 round bottom tubes. You would expect the results of the digest to be the same.

Student Workstation Setup

Note: There will be 5 student workstations. Each workstation aliquot will be enough for 4 colony cultures.

1. Prepare Plasmid Miniprep workstations using the following tables:

Consumable	# per Workstation
1.5 mL tubes	12
Miniprep Spin columns	4
Miniprep Collection tubes	4

Reagent	Volume per Workstation	# of 1.5 mL Tubes
Miniprep P1	1.1 mL	5
Miniprep P2	1.1 mL	5
Miniprep P3 (see note before preparation)	1.1 mL	5
Binding Buffer	1.2 mL	5
UltraPure Water	150 µL	5

Note: P3 Buffer must be chilled on ice for 30 minutes prior to use.

Note: Miniprep Wash 1 and Miniprep Wash 2 will also be needed for this reaction but should not be aliquoted due to it evaporating quickly.

PLASMID MINIPREP PROTOCOL

Note: Written for 1 blue and 1 white colony.

Bacterial Culture Preparation

1. Prepare (2) 1.5 mL tubes. Label one "blue" and the other "white"
2. Add 1 mL of the blue colony overnight to the tube labelled "blue"
3. Add 1 mL of the white colony overnight to the tube labelled "white"
4. Centrifuge bacterial cultures at max speed for 2 minutes
5. Discard as much of the supernatant as possible
6. Repeat steps 1–5 with the remaining 1 mL of each bacterial culture

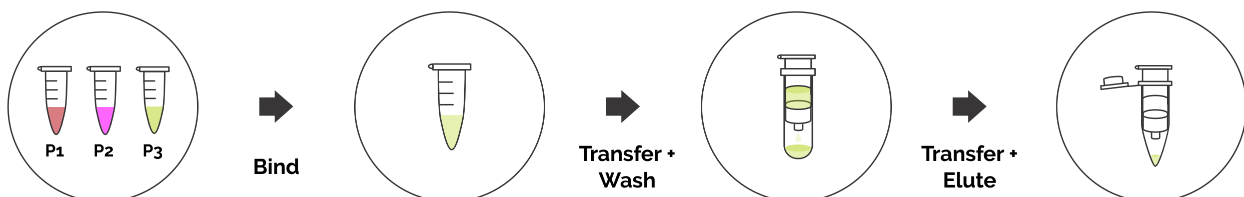
---

DNA Concentration

Note: Follow each of the steps below for both the blue and white samples, being careful not to mix the blue and white colony preparations.

7. Resuspend pellet completely in 250 μL Miniprep P1
8. Add 250 μL Miniprep P2 and immediately mix by inversion
   Note: The solution will go from a red to purple color.
9. Incubate at room temperature for 3 minutes
10. Add 250 μL of ice-cold Miniprep P3 and mix by inversion until purple coloring is completely changed to yellow
11. Incubate on ice for 5 minutes
12. Centrifuge for 5 minutes at 16,000 x g
13. Transfer 600 μL of the supernatant into a new 1.5 mL labeled microcentrifuge tube
14. Add 275 μL Miniprep Binding Buffer to the supernatant and mix by inversion
15. Transfer entire mixture from step 14 to a labeled miniprep spin column in a miniprep collection tube
16. Incubate for 2 minutes at room temperature
17. Centrifuge for 1 minute at 5,000 x g and discard flow through
18. Add 800 μL Miniprep Wash 1
19. Centrifuge for 1 minute at 5,000 x g and discard flow through
20. Add 800 μL Miniprep Wash 2
21. Centrifuge for 1 minute at 5,000 x g and discard flow through
22. Add 200 μL Miniprep Wash 2
23. Centrifuge for 1 minute at 5,000 x g and discard flow through
24. Centrifuge for 1 minute at 10,000 x g to remove any residual wash buffer
25. Transfer miniprep spin column to a new, labeled 1.5 mL microcentrifuge tube
26. Add 25 μL UltraPure Water directly to the filter
27. Incubate for 2 minutes at room temperature
28. Centrifuge for 1 minute at 10,000 x g

Note: This is the final edited DNA. It may be stored up to 1 month at –20˚C.



DOUBLE RESTRICTION DIGEST PREPARATION

Day of Double Restriction Digest Reaction

1. Prepare double digestion enzyme master mix
   a. To a 1.5 mL tube, add;
      - 144 µL of UltraPure Water
      - 48 µL of 10X Digestion Buffer
      - 24 µL of *Not*I enzyme
      - 24 µL of *Xmn*I enzyme
      - Mix well
   b. Aliquot (20) 0.2 mL tubes with 10 µL of master mix
2. Setup (5) 1.5 mL tubes with 30 µL of UltraPure Water each

Note: This will be given to the students as starting points for the experimental reaction.

3. Prepare agarose gel for gel electrophoresis
   a. Set up gel casting system to hold a 100 mL gel with a 15+ well comb
   b. To make 1 L of 1X TAE
      - To a 1 L glass bottle, add 980 mL of deionized water
      - Add 20 mL of 50X TAE to the bottle
      - Mix thoroughly
   c. To a 250 mL Erlenmeyer flask, add:
      - 1 g of Agarose
      - 100 mL 1X TAE
      - Swirl gently to mix
   d. Loosely ball up a paper towel in the top of the flask to prevent evaporation
   e. Microwave flask in 15–30 second intervals until agarose is fully dissolved
   f. Add 10 µL of SYBR™ Safe DNA Gel Stain to the agarose and swirl to mix
   g. Pour all 100 mL in the gel casting tray and allow to solidify
   h. Pour the remaining 1X TAE buffer into the gel electrophoresis chamber
      Note: Agarose gels may be prepared the night before and stored at 4˚C wrapped in saran wrap to prevent drying out, if needed.

DOUBLE RESTRICTION ENZYME DIGEST PROTOCOL

Note: Written for 1 blue and 1 white colony.

Reaction Setup	**Note:** Prepare on ice or cold block. Perform each step below for both the blue and white plasmid DNA. 1. To the labelled 0.2 mL tube containing the double digestion enzyme mix, add 0.25–0.5 µg (optimally) or 2–5 µL of purified DNA 2. Add UltraPure Water to bring to a final volume of 20 µL 3. Incubate at 37˚C for 1 hour 4. Add 3.3 µL of 6X Sample Loading Dye to all samples 5. Store on ice until ready to run agarose gel electrophoresis
Gel Electrophoresis	6. To the prepared 1% agarose gel, load; a. 5 µL of Diamond Ready-to-use DNA Ladder (0.1–10 Kbp) b. Up to 20 µL of double-digested DNA samples 7. Once all samples are loaded and the order recorded, run the gel for 1 hour at 100 volts 8. After electrophoresis is complete, visualize your gel

EXPECTED RESULTS

The CRISPR IN A BOX™ reaction allows for both phenotypic and genotypic readouts. The grown bacterial plates use blue/white screening based on the lacZ gene. Then, genotypic analysis may be done using a restriction enzyme digestion. Sanger sequencing may also be conducted to see the full scope of outcomes.

Bacterial Plates Example

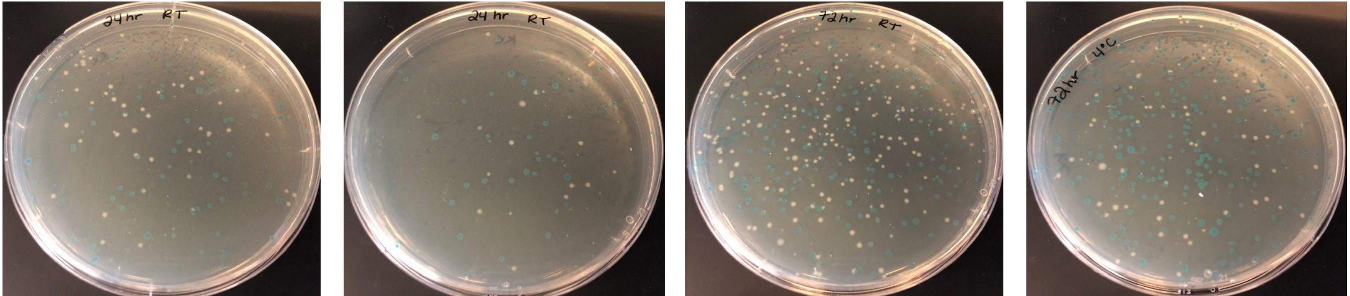


Figure 8: Gene editing reaction bacterial plates. Examples of the expected plates from the reaction. There should be a decent spread of both blue and white colonies. White colonies have had gene editing occur to some degree, while blue colonies are unedited.

Double Restriction Digestion Gel

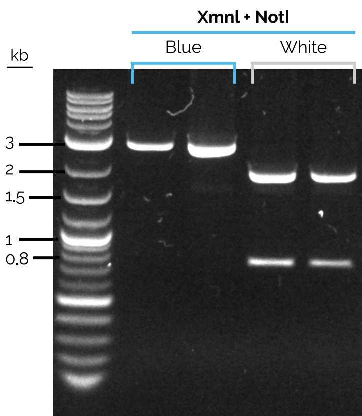


Figure 9: Restriction enzyme digestion on an agarose gel. Representation of an expected result gel from the restriction enzyme digestion. Blue and white colonies were digested with *Xmn*I and *Not*I restriction enzymes. The results from a plasmid digested with both enzyme yields two bands as a positive result.

Sanger Sequencing

INDEL —		% ▲	P-VALUE...	GCACTGGCCGTCG | TTTTACAACGTCGTGACTGGGAAAACCCTGGCGTTACCCAACTTAATCGCCTTGCAGCACA
HDR	8	40.2	0.00	GCACTGGCCGTCG | GCGGCCGCTTTTACAACGTCGTGACTGGGAAAACCCTGGCGTTACCCAACTTAATCGCCTT
WT	0	20.8	0.00	GCACTGGCCGTCG | TTTTACAACGTCGTGACTGGGAAAACCCTGGCGTTACCCAACTTAATCGCCTTGCAGCACA
G1 -3	-3	10.5	0.00	GCACTGGCCGTCG | ---TACAACGTCGTGACTGGGAAAACCCTGGCGTTACCCAACTTAATCGCCTTGCAGCACA
G1 -2	-2	8.2	0.00	GCACTGGCCGTC- | -TTTACAACGTCGTGACTGGGAAAACCCTGGCGTTACCCAACTTAATCGCCTTGCAGCACA
G1 -12	-12	8.1	0.00	GCACTGGC----- | -------ACGTCGTGACTGGGAAAACCCTGGCGTTACCCAACTTAATCGCCTTGCAGCACA
G1 +1	1	6.8	0.00	GCACTGGCCGTCG | CTTTTACAACGTCGTGACTGGGAAAACCCTGGCGTTACCCAACTTAATCGCCTTGCAGCAC
G1 +3	3	5.4	0.00	GCACTGGCCGTCG | TAGTTTTACAACGTCGTGACTGGGAAAACCCTGGCGTTACCCAACTTAATCGCCTTGCAGC

Figure 10: Simulated Sequencing Output for 1364-NS HDR Reaction. Representation of a few possible sequencing outcomes from the CRISPR IN A BOX reaction. The red box is capturing the colonies that were edited perfectly. 40% of the colonies sequenced had this correction pattern. The remaining sequences illustrate deletions and insertions that may occur. Both insertions and deletions have the ability to turn a colony white.

BUFFER PREPARATION GUIDE

Note: All the buffers are included in the kit. The following instructions are for user reference.

10X Hypotonic Buffer

Reagents	Preparation (10 mL)	Final Concentration (mM)
1M TRIS (pH 7.0)	2 mL	200 mM
1M $MgCl_2$	1.5 mL	150 mM
DI Water	6.5 mL	-

Store in 4˚C fridge until use.

10X *In Vitro* Buffer

Reagents	Preparation (1 mL)	Final Concentration (mM)
10X Hypotonic Buffer	896 µL	-
1M DTT	4 µL	4 mM
100 mM ATP	100 µL	10 mM

Use immediately.

Cas Buffer

Reagents	Preparation (10 mL)	Final Concentration (mM)
1M HEPES (pH 7.5)	200 µL	20 mM
1M KCl	1.5 mL	150 mM
DI Water	8.3 mL	-

Store at room temperature.

RNA Buffer

Reagents	Preparation (10 mL)	Final Concentration (mM)
1M HEPES (pH 7.5)	300 µL	30 mM
1M Potassium Acetate	1 mL	100 mM
DI Water	8.7 mL	-

Store at room temperature.

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