Edvo-Kit # 307

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Code Breakers: Using CRISPR to Rewrite Genetics

Experiment Objective:

In this experiment, students explore cutting edge genetic engineering using CRISPR-Cas9 to knock out a protein coding gene in classroom safe bacteria. In addition, they'll learn to design and screen gRNAs using base paring rules, probability, and bioinformatics.

See page 3 for storage instructions.

Version 307.240424

EDVOTEK®

Table of Contents

Safety Data Sheets can be found on our website: www.edvotek.com/safety-data-sheets

Experiment Components

Components	Storage	Check (✓)
• LyphoCells™ *E. coli* Purple	-20°C Freezer	❑
• IPTG	-20°C Freezer	❑
• Chloramphenicol	-20°C Freezer	❑
• Ampicillin	-20°C Freezer	❑
• pCas9-PurpleGuide plasmids	-20°C Freezer	❑
• pCas9-ControlGuide plasmids	-20°C Freezer	❑
• $CaCl_2$	-20°C Freezer	❑
• Competent Cell Solution	-20°C Freezer	❑
• SOC Liquid Media (broth)	-20°C Freezer	❑
• SOC Additive (for ReadyPour™ ONLY)	-20°C Freezer	❑

This experiment is designed for 10 groups.

Sample volumes are very small. It is important to quick spin the tube contents in a microcentrifuge to obtain sufficient volume for pipetting. Spin samples for 10-20 seconds at maximum speed.

IMPORTANT READ ME!

This experiments contain antibiotics which are used for the selection of transformed bacteria. Students who have allergies to antibiotics such as penicillin, ampicillin, kanamycin, tetracycline, and chloramphenicol should not participate in this experiment.

Reagents & Supplies

Store components below at Room Temperature.

- Bottle of ReadyPour™ SOC Agar, sterile ❑
- Petri plates, small ❑
- Petri plates, large ❑
- Wrapped 10 mL pipet, sterile ❑
- Inoculating loops ❑
- Microcentrifuge tubes ❑
- 50 mL Conical tubes ❑

Requirements *(These items are NOT included in the experiment)*

- Adjustable Volume Micropipettes and tips
- Two Water Baths (37°C and 42°C)
- Floating racks or foam tube holders
- Thermometer
- Incubation Oven (37°C) (highly recommended but optional)
- Vortexer (optional)
- Centrifuge(s)
- Ice Buckets and Ice
- Marking pens
- Tape
- Pipet pumps or bulbs
- Microwave
- Hot gloves

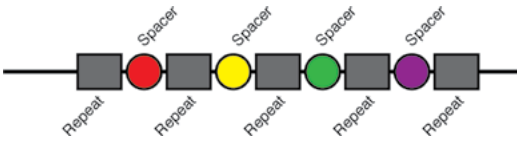
Background Information

CRISPR is a game-changing gene-editing tool that is catalyzing a wave of innovations across fields as diverse as biotechnology, medicine, agriculture, and energy. This system evolved in bacteria at the beginning of evolutionary history as a defense against viral attacks and was discovered by humans in the late 1980s. However, it would take scientists another 20 years to harness CRISPR as a precise and versatile gene editor.

Today, CRISPR's unmatched ability to rewrite the code of life has captured the imagination of researchers and the public alike. CRISPR technologies are used to cure diseases like cancer and sickle cell anemia, develop antibiotics, study complex genetic traits, understand evolutionary history, create drought-resistant crops, and develop new materials like bioplastics and biofuels. As CRISPR research continues to advance its transformative potential will be limited only by our imagination and by the regulations we create.

Where Does the Name CRISPR Come From?

CRISPR, short for Clustered Regularly Interspaced Short Palindromic Repeats, describes uniquely patterned regions of bacterial DNA. An enzyme tightly linked to these CRISPR region was identified by scientists and called Cas for "CRISPR-associated proteins". In bacteria and archaea, Cas enzymes cut and destroy foreign DNA, guided by the nucleotide sequences stored in CRISPR regions. Cas enzymes are central to new DNA editing technologies but the CRISPR region itself is not. Never the less the CRISPR name has become synonymous with these technologies.



EXPLORING THE CRISPR-Cas SYSTEM

At the heart of CRISPR gene editing are two molecules: an enzyme called Cas (Figure 1a) and a piece of RNA known as guide RNA or gRNA (Figure 1b). These molecules collaborate to form a complex capable of identifying and cutting a specific DNA sequence within a genome, known as the target DNA. Following a cut, a cell's intrinsic repair mechanisms mend the targeted DNA often with the help of additional DNA instructions, called template DNAs, that the experimenter provides. This repair process leads to changes in the DNA sequence that can induce a loss of function mutation, correct a mutation, or introduce a new trait in an organism.

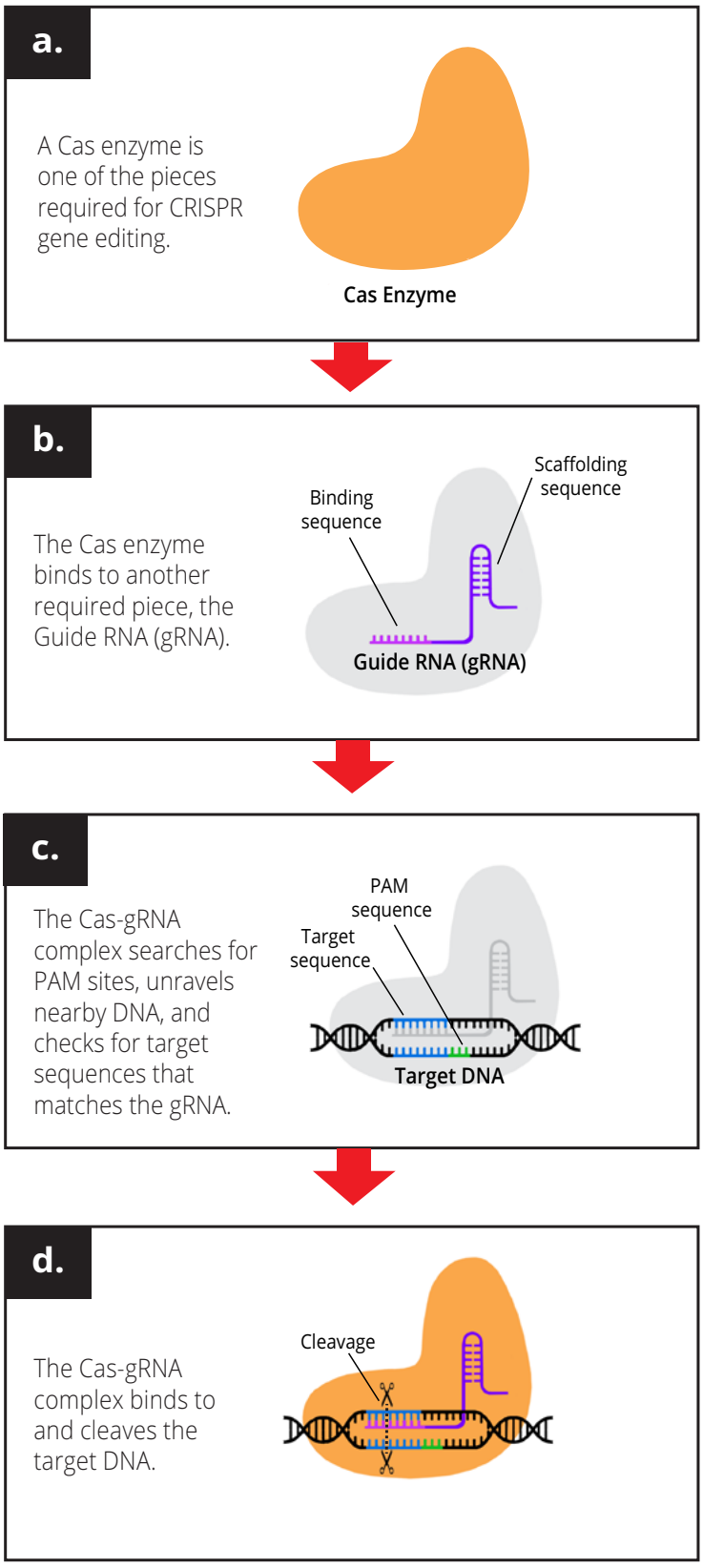
What's the difference between Cas and Cas9?

Multiple Cas (CRISPR associated proteins) nucleases have been identified. Cas9 is a prominent example. Originally discovered in *Streptococcus pyogenes*, Cas9 quickly gained popularity in CRISPR gene editing due to its high efficiency and accuracy. Other commonly used CRISPR endonucleases include Cas3, Cas12, Cas13, SuperFi-Cas9, and Cs7-100.

Cas proteins are endonucleases. These are enzymes that cut DNA by breaking the phosphodiester bonds between nucleotides. Some endonuclease cut DNA non-specifically, but most cleave at specific nucleotide sequences called target sites. Cas enzymes fall into this latter category. They target specific sites within a DNA molecule and create a double-strand break. However, unlike most endonucleases (like restriction enzymes), which have a target DNA sequence built into their structure, CRISPR outsources its specificity to interchangeable molecules of guide RNA. This means that Cas enzymes can be programmed to cut any DNA region by creating guide RNA molecules that are complementary to the target's sequence.

Guide RNAs (gRNA), sometimes called single guide RNA (sgRNA), are the second key component for CRISPR gene editing. Serving as 'molecular guides', they direct the Cas enzymes to a specific target site within a genome. Guide RNA molecules consist of two functional parts. The first is a scaffolding sequence (Figure 1b) where the Cas enzyme binds. The second is an approximately 20-nucleotide sequence which determines where the DNA will be cut (Figure 1b). Through base-pairing interactions, this nucleotide sequence aligns with the target DNA and brings the Cas enzyme to the intended cut site (Figure 1d).

Figure 1: The CRISPR-Cas System



To ensure proper interactions between the CAS-gRNA complex and the target DNA, there's an additional requirement: a nearby DNA sequence known as a **P**rotospacer **A**djacent **M**otif, or PAM, sequence in the organism's DNA (Figure 1c). This is a sequence of two to six nucleotides found immediately downstream of the DNA sequence targeted by the gRNA. PAM sequences allow the Cas enzyme to properly bind to, scan, and cut DNA. However, they also limit what DNA regions can be targeted. For example, the popular CRISPR enzyme Cas9 requires a 5'-NGG-3' PAM sequence (where "N" can be any nucleotide base). This means that for a researcher using Cas9 the genomic locations that can be targeted are restricted to those containing a downstream 5'-NGG-3' sequence. Luckily, there are a range of Cas enzymes each with its own unique PAM sequences.

The target DNA (Figure 1c) represents the final critical component for the CRISPR experiment. This is the specific sequence of DNA that researchers aim to modify. During a CRISPR experiment, the Cas-gRNA complex scans an organism's DNA by unwinding short segments of DNA and comparing these sequences to the gRNA's 20 bp binding sequence. When the complex reaches a region of DNA that is complimentary, it attaches. Once attached the Cas enzyme cuts the DNA (Figure 1d).

THE FORK IN THE ROAD: HOMOLOGOUS DIRECT REPAIR AND NON-HOMOLOGOUS END JOINING

Once a Cas enzyme has cut through the double stranded DNA, the cell fixes the damaged nucleic acid chain using its intrinsic DNA repair pathways. Researchers design their CRISPR experiments so that the cell repairs the DNA in one of two ways: using **N**on-**H**omologous **E**nd **J**oining (NHEJ) or **H**omologous **D**irect **R**epair (HDR). These two repair mechanisms produce different results, allowing researchers to change the target DNA in distinct ways.

NHEJ (Figure 2) is a simple but error-prone process that occurs naturally in cells and is used in the lab for knock-out experiments. These are experiments that study the function of a gene by inactivating it with a loss of function mutation and then observing the results. When Cas cuts through the DNA backbone, the cell's most direct means of repair is to join the two broken ends of DNA back together. However, this process usually results in small deletions or insertions at the cut site which cause frameshift mutations that disrupt the function of the targeted region. Unlike classical knockout methods, which required screening thousands of random mutations, CRISPR knockout experiments allow researchers to design guide RNA that automatically targets their gene of interest, streamlining the process by eliminating one of the most laborious and time-intensive steps.

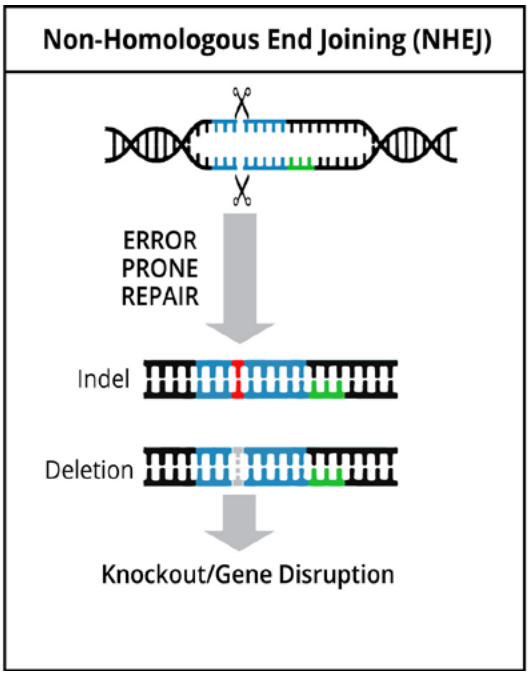


Figure 2: NHEJ

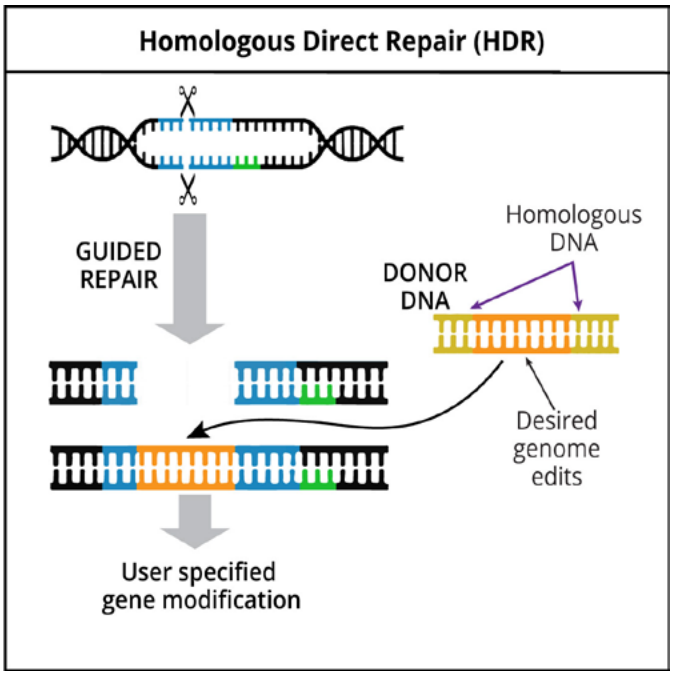


Figure 3: HDR

HDR is also a naturally occurring process but more precise, controlled, and complex than NHEJ (Figure 3). It involves mending the double-stranded break using a donor or template DNA as a reference. To work properly, this template DNA must contain regions that match the target DNA sequences on both sides of the break. In cells, this is often the sister chromosome, which shares the same sequence. In CRISPR experiments, researchers develop a repair template that matches the genomic DNA around the cut site. However, in between these homologous beginning and ending regions, scientists can introduce specific mutations or new genes. As the cell repairs itself, these insertions are transcribed from the template to the target DNA allowing for specific and user-designed changes to an organism's genome.

THE CRISPR ADVANTAGE: PRECISE, SIMPLE, UNIVERSAL

CRISPR has quickly surpassed previous gene editing technologies like restriction enzymes, zinc finger nucleases, and TALENS in popularity and power because of its unprecedented advantages. Chief among these are precision, simplicity, and universality.

Precision: CRISPR technology allows for highly targeted gene editing. That's because gRNA contains around 20 base pairs that must match sequentially with the nucleotides in the experimental DNA. The chance of this happening is 4^{20} or 1 in every 1,099,511,627,776 base pairs! For reference, the human genome's size is ~6,270,000,000 base pairs. This means that, in the average human genome (or any other similarly sized genome), the chance of having additional, unplanned cut sites is incredibly low. Contrast this with most restriction enzymes, which have recognition sites between 4 and 8 bp, and thus a much higher likelihood of cutting genomic DNA at multiple locations.

Simplicity: Designing and synthesizing gRNA is fast and cost-effective. Previously, highly specific enzyme "scissors" (like zinc finger nucleases and TALENS) involved an incredible amount of effort and capital to create, limiting where and how they could be used. In contrast, creating an effective Cas-gRNA system is simple, affordable, and fast. For example, it's now possible to create giant libraries of guide RNAs that target every gene in a genome!

Universality: While the Cas guide RNA system evolved in bacteria, it has been adapted to work in most eukaryotic cells. This versatility, combined with the system's ease of use, means that genetic experiments and questions previously limited to just a few model organisms can now be tackled in a diverse array of organisms and by a much wider network of researchers.

Given this combination, it's no wonder that CRISPR is a game-changer!

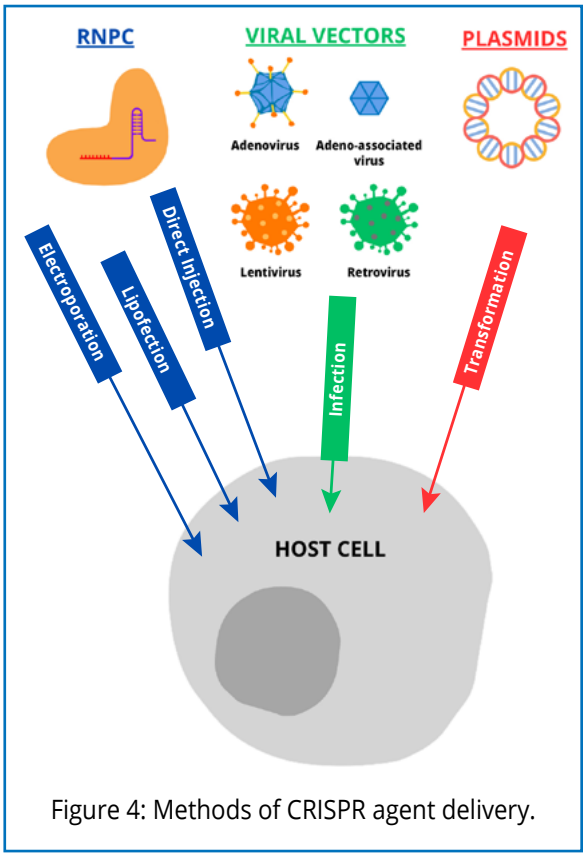


Figure 4: Methods of CRISPR agent delivery.

PUTTING IT IN A CELLULAR CONTEXT

Up until now, we've focused on the key molecules involved in CRISPR technologies - the Cas enzymes, guide RNAs, target DNAs, etc. However, unlike the word document editing that this technology is often compared to, CRISPR is much more than a change of As, Ts, Gs, and Cs. CRISPR's precise gene editing is a complex process that occurs within living cells and organisms.

One of the key hurdles scientists faced when transforming CRISPR-Cas from a defense mechanism found in bacteria to a world-changing DNA editing tool was finding a way to deliver the Cas enzyme, gRNA, and template DNA into cells. Luckily, researchers have developed several strategies to accomplish this (Figure 4).

One approach is the direct delivery of Cas and gRNA molecules into cells as a combined unit called a ribonucleoprotein (RNP) complex. RNP complexes can be introduced into cells through techniques such as electroporation (increasing a cell's permeability using electrical pulses) and lipofection (coating the complex so that it can fuse with the cell's membrane), or by using gold nanoparticles as carriers.

Another approach involves delivering the necessary *genetic information* for the creation of Cas enzymes, gRNAs, and other needed components into the cells. In this scenario, the cells' own machinery then reads these introduced genes and produces the corresponding components. Scientists commonly use viral vectors, modified viruses that can target specific cells and infect them with customized RNA, to insert genetic material. For bacterial, yeast, fungal, and certain plant research, plasmids are also popular. These circular, extrachromosomal DNA strands can be introduced to a cell through a process known as transformation. They are favored in CRISPR experiments because they are capable of self-replication, well-received by many cells, and large enough to accommodate the insertion of genes coding for the gRNA, Cas enzyme, and (if needed) the template DNA.

However they arrive within the cell, CRISPR components work together to modify the targeted DNA sequence, ultimately leading to phenotypic changes. This complex process is summarized by the central dogma of molecular biology (genetic information flows only in one direction, DNA-->RNA-->protein). However, in the dynamic environment of a living cell, this process is anything but straightforward. It involves many intricate and sometimes unexpected variations that occur during transcription, translation, protein folding, protein interactions within the cell, and interactions between cells in the organism.

Given the internal and environmental variables involved, how can researchers analyze and evaluate the success of their CRISPR experiment? Molecular techniques such as PCR and sequencing can confirm that the DNA has been targeted and correctly modified. However, the ultimate test of the experiment's success is how the mutation plays out within a cell or organism. *In vivo* screenings for specific phenotypes provide the most accurate representation of the experiment's success by providing information about how gene modification has impacted the organism's health and new capabilities.

APPLICATIONS AND ETHICS

Just as the impacts of DNA changes are best viewed in the larger context of a cell or organism, the power of CRISPR is also best viewed in a larger context. CRISPR is an incredibly powerful gene editing tool that can be used to investigate gene function, modify a genome, and engineer cells or organisms with specific properties. This gives CRISPR enormous potential for improving things that we value and need such as human health, the environment, and scientific understanding (see page 12.) At the same time, the ethical implications of CRISPR are complex and should be carefully considered. These are the same for all gene modification technologies but the efficiency of CRISPR has made addressing them far more urgent. As CRISPR becomes more common and its applications expand, scientists, policymakers, and citizens must consider the technology's social implications (good and bad) and ethical questions.

One of the most controversial issues is the use of CRISPR for germline editing in humans. Editing human embryos, sperm, or eggs could be used to cure genetic diseases or prevent them from being passed to future generations but also raises concerns about eugenics, discrimination based on genetic traits, and designer babies. There are also biological concerns such as non-target edits. For example, although the CAS cleavage is incredibly specific, it's still possible for the enzyme to digest unexpected sites within the genome. This is a particularly serious concern in gene therapies.

> "I think it's good to be reminded that we are in this very fortunate position to be able to make a positive change, and we shouldn't screw it up...We all have a responsibility: scientists, media, policymakers, and bioethicists have an obligation to participate in the discussion. I think as scientists we can help convey what is the technology, help explain what it is, and to understand what the technology's potentials are."
> - Dr. Feng Zhang , molecular biologist and CRISPR pioneer

As CRISPR democratizes gene editing, there's also a growing concern that individuals might intentionally introduce hazardous mutations into organisms, such as viruses or bacteria, for nefarious purposes. At the ecosystem level, there are concerns that edited genes could spread to wild populations.

These risks and ethical considerations must be carefully considered and planned for by a public that understands the biology, the potential, and the need for limits on this powerful tool.

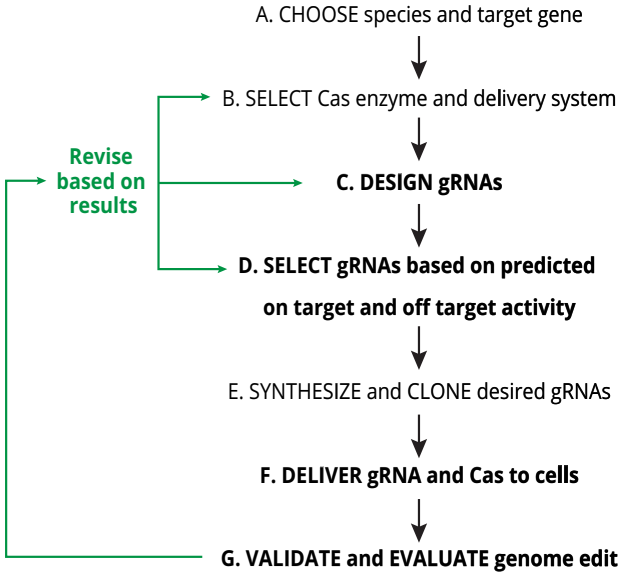


Figure 5: Key Steps in a CRISPR Experiment
(Steps in bold are part of this lab's activities)

Your Experiment

While the CRISPR research landscape is remarkably diverse, most experiments follow a general execution model (Figure 5). In this experiment, you will undertake several critical steps within this model.

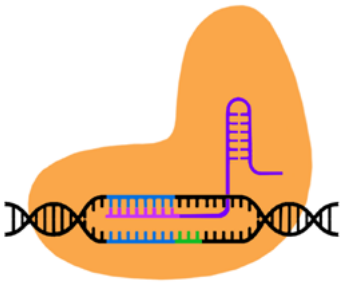
In Module I, you will design gRNAs targeting the LacZ gene in *Escherichia coli* (*E. coli*). The LacZ gene encodes the enzyme β-galactosidase and is frequently used as a reporter gene in molecular biology. The β-galactosidase enzyme, often called beta-gal, cleaves X-gal, a colorless substrate, resulting in a blue product. This turns bacteria colonies blue indicating the presence of the gene and enzyme. When designing gRNA, you create an RNA sequence that can theoretically guide the Cas protein to a specific genomic location. Effective gRNAs are complementary to a target DNA sequence, contain a nearby PAM site, and are unique within the genome. To achieve this, you will design gRNAs based on a partial sequences of *E. coli's Lac*Z gene. Using this DNA sequence allows you to choose a target sequence that is directly upstream of a PAM site and to design a gRNA that precisely base-pairs with the target. You will also predict potential off-target effects using probability, with the option for an additional bioinformatics analysis (Appendix B).

What is a Chromoprotein?

Chromoproteins are naturally pigmented due to the presence of a chromophore within its structure. The chromophore is a region of secondary structure that strongly absorbs visible light and gives off a distinct color in ambient light. While these proteins belong to the fluorescent protein family, they can be detected by the naked eye, unlike most fluorescent proteins. This ability for instrument-free detection makes them a popular molecular biology tool.

In Module II you will use a CRISPR knockout protocol to target a purple chromoprotein gene in a strain of classroom-safe *E. coli* bacteria. During the experiment, you will transform competent bacterial cells with a plasmid containing three key components: the Cas9 enzyme gene, a guide RNA (gRNA) that targets the purple chromoprotein gene, and a chloramphenicol resistance gene. Once the plasmid is inside the cells, the Cas9 and chloramphenicol resistance genes are transcribed and translated into proteins by the bacterial machinery. The chloramphenicol resistance protein enables the transformed cells to survive on chloramphenicol plates, while non-transformed cells are killed by the antibiotic. The transcribed gRNAs associate with the Cas9 proteins to form functional CRISPR complexes. These complexes, guided by their gRNAs, seek out and bind to the purple chromoprotein gene sequences. If the target sequence is present, Cas9 cuts the DNA, triggering a non-homologous end joining (NHEJ) repair process that inactivates the gene. This results in an observable phenotypic change in the bacterial colonies, turning them from purple to white (or light purple).

Study Questions (Answer at any time)



1. Label the following CRISPR components: Cas enzyme, guide RNA, PAM site, and target DNA. Next, in one sentence or less, describe their role during a CRISPR experiment.

2. You are designing a CRISPR system that converts a Guanine (G) to a Cytosine (C) at the mutation site. List at least three essential CRISPR components necessary for achieving this targeted genetic correction. Bonus: Propose a method to get these components into a cell.

3. Write an "H" (for HDR) or an "N" (for NHEJ) on the line for each statement depending on which process the sentence best describes.

   a. _____ Simple but error-prone repair process
   b. _____ Precise, controlled, and complex repair process
   c. _____ Popular for targeted knock-out experiments
   d. _____ Repair process that often results in small deletions or insertions
   e. _____ Cell repairs DNA using a reference DNA strand
   f. _____ Cell repairs DNA by joining the two broken ends back together
   g. _____ Used to introduce user-designed changes to an organism's genome
   h. _____ Uses sister chromosome or user designed donor DNA as template

4. Briefly describe one key advantage of CRISPR over prior gene editing methodologies.

5. Research and briefly describe a specific application of CRISPR technology. (Hint: Consider biomedicine, agriculture, synthetic biology, conservation, and energy.)

6. Why is CRISPR sometimes compared to word processing? Bonus: Come up with an another appropriate metaphor for CRISPR and describe it below.

7. CRISPR is: _____

   A. An enzyme that synthesizes DNA or RNA molecules by assembling nucleotides in a sequence-specific manner.
   B. Uniquely patterned regions of bacterial DNA involved in defense.
   C. The name given to a new type of gene editing.
   D. Answers a and b.
   E. Answers b and c.

Study Questions (Answer at any time)

8. What is the primary function of the CRISPR in nature? ____
   A. To produce energy for the cell.
   B. To repair DNA damage.
   C. To defend bacteria against viruses.
   D. To regulate gene expression.
   E. All of the above.

9. Which enzyme is responsible for cutting the DNA in CRISPR systems? ____

   A. DNA polymerase.
   B. Cas9 only.
   C. Cas9 and other Cas enzymes.
   D. Restriction enzymes.
   E. Guide enzymes.

10. What type of genetic alterations is CRISPR used to perform? ____

    A. Small insertions or deletions (indels) that result in a gene knock-out.
    B. The insertion of a specific DNA sequence into a particular location in the genome (sometimes called a knock-in).
    C. Single base changes to correct a point mutation.
    D. Single base changes to introduce a new mutation.
    E. All of the above.

CHALLENGE QUESTION: How do you think CRISPR technology can best be discussed by society? Why are these discussions important? What can you do to engage people in such discussions.

5 AMAZING CRISPR POWERED INNOVATIONS

01

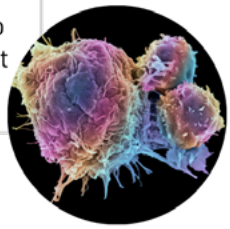
New Gene Therapies

Hailed as the beginning of the end for genetic diseases, CRISPR therapy treats sickle cell disease. Scientists are also working on curing antitrypsin deficiency, hereditary angioedema, Huntington's disease, muscular dystrophy, and other inherited conditions.

02

Cancer Immunotherapy

CRISPR-Cas9 has been used to engineer T-cells that can target and kill cancer cells.



03

Improved Crops

CRISPR-Cas9 has been used to develop crops with enhanced yields, increased resistance to pests and diseases, and improved nutritional profiles.

04

New Models

CRISPR-Cas9 has been used to create diverse model organisms, enabling researchers to better understand diseases, develop new treatments, and study evolution and biology. For example, researchers used CRISPR to study the genetic basis of social behavior in ants.

05

New Materials

CRISPR-Cas9 has been used to engineer bacteria capable of producing new materials, such as biodegradable plastics and algae capable of producing economically viable and robust biofuels.

For more CRISPR-enabled innovations,
CLICK HERE!
https://blog.edvotek.com/?s=crispr

Experiment Overview

EXPERIMENT OBJECTIVE:

In this experiment, students explore cutting edge genetic engineering using CRISPR-Cas9 to knock out a protein coding gene in classroom safe bacteria. In addition, they'll learn to design and screen gRNAs using base paring rules, probability, and bioinformatics.

LABORATORY NOTEBOOKS:

Scientists document everything that happens during an experiment, including experimental conditions, thoughts and observations while conducting the experiment, and, of course, any data collected. Today, you'll be documenting your experiment in a laboratory notebook or on a separate worksheet.

Before starting the Experiment:
- Carefully read the introduction and the protocol. Use this information to form a hypothesis for this experiment.
- Predict the results of your experiment.

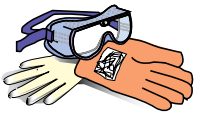
During the Experiment:
- Record your observations.
- Record any challenges faced while performing the experiment.

After the Experiment:
- Interpret the results – does your data support or contradict your hypothesis?
- If you repeated this experiment, what would you change? Revise your hypothesis to reflect this change.

Laboratory Safety

IMPORTANT READ ME!

This experiments contain antibiotics which are used for the selection of transformed bacteria. Students who have allergies to antibiotics such as penicillin, ampicillin, kanamycin, tetracycline, and chloramphenicol should not participate in this experiment.



Wear gloves and safety goggles

1. Wear gloves and goggles while working in the laboratory.
2. Exercise extreme caution when working in the laboratory - you will be heating and melting agar, which could be dangerous if performed incorrectly.
3. DO NOT MOUTH PIPET REAGENTS - USE PIPET PUMPS OR BULBS.
4. The *E. coli* bacteria used in this experiment is not considered pathogenic. Regardless, it is good practice to follow simple safety guidelines in handling and disposal of materials contaminated with bacteria.
   A. Wipe down the lab bench with a 10% bleach solution or a laboratory disinfectant.
   B. All materials, including petri plates, pipets, transfer pipets, loops and tubes, that come in contact with bacteria should be disinfected before disposal in the garbage. Disinfect materials as soon as possible after use in one of the following ways:
   - Autoclave at 121°C for 20 minutes.
     Tape several petri plates together and close tube caps before disposal. Collect all contaminated materials in an autoclavable, disposable bag. Seal the bag and place it in a metal tray to prevent any possibility of liquid medium or agar from spilling into the sterilizer chamber.
   - Soak in 10% bleach solution.
     Immerse petri plates, open tubes and other contaminated materials into a tub containing a 10% bleach solution. Soak the materials overnight and then discard. Wear gloves and goggles when working with bleach.
5. Always wash hands thoroughly with soap and water after working in the laboratory.
6. If you are unsure of something, ASK YOUR INSTRUCTOR!

Module I-A: Designing gRNA

The key to CRISPR technology's power, precision, and accessibility are its customizable guide RNA molecules whose 20-base pair sequences are designed to target a user-specified place in the genome (see box "Looking at DNA, Making RNA".) In this module, you will design five candidate gRNAs that target the LacZ gene.

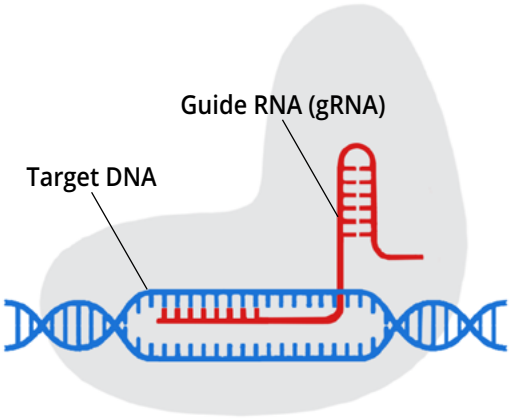
The LacZ gene codes for the enzyme β-galactosidase and is found in many enteric bacteria. As part of the lac operon, β-galactosidase aids in bacterial metabolism by breaking down lactose when glucose is not available. In the lab it is a tool for CRISPR, cloning, and other genetic engineering experiments.

In research labs, this versatile tool cleaves X-gal which produces a bright blue product. For instance, LacZ is used in blue-white screening, where genes are cloned into a site located in the middle of the gene coding region. Plasmids with successful inserts have disrupted the LacZ gene and the colonies remain white. In contrast, those without inserts turn blue, making it easy for scientists to identify recombinant clones.

In *E. coli*, the LacZ gene is 3071 bp. However, in this exercise you will focus on the first 200 nucleotides to design five candidate gRNAs for a knockout experiement targeting the LacZ gene.

Looking at DNA, Making RNA

When designing guide RNA (gRNA) for CRISPR, researchers look at DNA sequences because the CRISPR-Cas system targets DNA (often genes). However, the guide RNA that directs the Cas enzyme to the desired DNA sequence is RNA. How does this work? RNA can bind to DNA through a process called RNA-DNA hybridization. This occurs when the nucleotide sequences of RNA and DNA are complementary to each other, allowing them to form hydrogen bonds between their bases. The binding is facilitated by similar base-pairing rules as in DNA-DNA interactions, namely A-U (adenine-uracil) and G-C (guanine-cytosine) base pairing. During the first stages of a CRISPR experiment, gRNAs try to pair with short sequences along recently unwound DNA. If the gRNA sequence and the target DNA sequence are fully complimentary, a match occurs and the gRNA temporarily hybridizes to a strand of DNA. This binding allows the Cas enzyme to bind to and cleave the DNA which in turn begins the process of repair and gene editing.



Module I-A: Designing gRNA, continued

TO DESIGN CANDIDATE gRNAs:

1. **IDENTIFY & HIGHLIGHT** five PAM sites in the target sequence. For this experiment, assume that you are using a Cas9 enzyme which uses an 5'-NGG-3' PAM site. In this notation, the "N" can be any nucleotide. This means that the Cas9 will only bind to sequences immediately *upstream* (in the 5′ direction) of an AGG, TGG, CGG, or GGG sequence.

EXAMPLE SEQUENCE:

```
5′-GCGTTCAGCAAAAAGTGAATTCTTGGTTA-3'
3′-CGCAAGTCGTTTTTCACTTAAGAACCAAT-5'
```

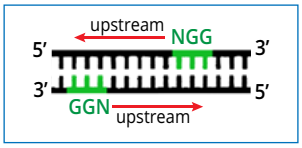
IDENTIFY & HIGHLIGHT

```
5'-GCGTTCAGCAAAAAGTGAATTCTTGGTTA-3'
3′-CGCAAGTCGTTTTTCACTTAAGAACCAAT-5'
```

2. **IDENTIFY & UNDERLINE** the binding regions upstream of your highlighted PAM sites. In CRISPR, the guide RNA recognizes and binds to 20 nucleotides on the DNA strand *opposite* from the NGG PAM site. ***Remember, binding always occurs upstream (in the 5′ direction) of the PAM sites.***

IDENTIFY & UNDERLINE

```
5′-GCGTTCAGCAAAAAGTGAATTCTTGGTTA-3'
3′-CGCAAGTCGTTTTTCACTTAAGAACCAAT-5'
```



3. Using base pairing rules, **CREATE A GUIDE SEQUENCE** for the gRNA. This sequence should bind to the underlined region identified in step 2. For this DNA-RNA pairing (see box "Looking at DNA, Making RNA") Adenine (A) pairs with Uracil (U), Thiamine (T) binds with Adenine (A), Cytosine (C) pairs with Guanine (G), and Guanine (G) to Cytosine (C). ***Note that for a PAM site on the bottom strand, a 5′ to 3′ direction means recording the sequence from right to left.***

CREATE A GUIDE SEQUENCE

```
AAGTCGTTTTTCACTTAAGA
|||||||||||||||||||| (guide sequence)
UUCAGCAAAAAGUGAAUUCU
```

4. **USING** steps 1-3 from page 15, **DESIGN** five candidate gRNAs for the *Lac*Z Gene Sequence (0-200 bp). **RECORD** your answers in Table 1.

There are many candidate gRNA that can be designed from this sequence. The five you choose and design may be different from your classmates.

continued

Module I-A: Designing gRNA, continued

_Lac_Z Gene Sequence (0-200 bp)

```
5′–  ATGACCATGATTACGGATTCACTGGCCGTCGTTTTACAACGTCGTGACTG
3′–  TACTGGTACTAATGCCTAAGTGACCGGCAGCAAAATGTTGCAGCACTGAC

     GGAAAACCCTGGCGTTACCCAACTTAATCGCCTTGCAGCACATCCCCCTT
     CCTTTTGGGACCGCAATGGGTTGAATTAGCGGAACGTCGTGTAGGGGGAA

     TCGCCAGCTGGCGTAATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAA
     AGCGGTCGACCGCATTATCGCTTCTCCGGGCGTGGCTAGCGGGAAGGGAA

     CAGTTGCGCAGCCTGAATGGCGAATGGCGCTTTGCCTGGTTTCCGGCACC – 3′
     GTCAACGCGTCGGACTTACCGCTTACCGCGAAACGGACCAAAGGCCGTGG – 5′
```

NOTE: Since Cas9 can bind to either of the complementary DNA strands, remember to examine both for PAM sequences. The PAM sequence on the lower strand will be "GGN" because this strand has a 3' to 5' direction.

TABLE 1

gRNA Name	Guide Sequence (RNA)	PAM Sequence (DNA)
gRNA #1		
gRNA #2		
gRNA #3		
gRNA #4		
gRNA #5		

Module I-B: Determining Specificity

One concern of gene editing is off-target effects. In CRISPR, off-target effects occur when Cas acts on un-targeted genomic sites and create additional cut sites. These can lead to genetic modifications in locations other than the intended one, potentially causing disruptions to normal gene function and cellular processes. To minimizing off-target effects researchers use a combination of computational predictions and experimental validations to determine the specificity of their gRNA.

In this exercise, you will use the rules of probability (see "IMPORTANT INFORMATION" box) to estimate the chance that a gRNA you designed in Part A will have off target sites.

1. **DETERMINE** the probability that a nucleotide is a certain base (such as a "T"). Remember that probability equals the number of favorable outcomes (the nucleotide is that particular base) divided by the total number of possible outcomes (the number of different nucleotides found in DNA). ***EXAMPLE:The probability that you will flip a coin and get heads is 1 (1 head side) divided by 2 (two possible sides). This is 1/2 or 0.5.***

2. **DETERMINE** the probability of finding the entire guide sequence of one of your gRNAs using your answer from questions 1 and the multiplication rule of probability. For this exercise assume that every nucleotide position has an equal probability of being A, T, C, or G and assume that each match is an independent event. ***EXAMPLE: The probability of flipping a coin four times and getting a sequence of heads, tails, tails, heads is 1/2 * 1/2 * 1/2 * 1/2 = 0.0625.***

3. **DETERMINE** the probability that following the target sequences is the PAM sequence of NGG again using the multiplication rule of probability. Remember that N can be any nucleotide. ***EXAMPLE: The probability of flipping a coin four times and getting the above sequence and then flipping a coin three times and getting either head or tails, then tails, then tails again is 0.0625 * 1 * 1/2 * 1/2 = 0.015625.***

IMPORTANT INFORMATION

- Probabilities fall between 0 and 1.
- The Probability Rule of Multiplication states that:
  - If A and B are two independent events in a probability experiment, then the probability that both events occur simultaneously is: P(A and B)=P(A) • P(B)
  - In case of dependent events, the probability that both events occur simultaneously is: P(A and B)=P(A) • P(B|A) (The notation P(B|A) means "the probability of B, given that A has happened.")
- *E. coli* genome's sizes range from 4.5 MB to 5.5 MB. Assume 5 MB (5,000,000 bp) for this module.

Module I-B: Determining Specificity, continued

4. **MULTIPLY** the probability you determined in step 3 by the size of the genome of your target organism in this case *E. coli*. This is the number of potential off-target sites in *E. coli* for your first gRNA. ***EXAMPLE: Pretend the probability you determined in step 3 was 0.015625 and your target organisms genome size was 200. The number of potential off target sites would be 0.015625*200 = 3.124 sites.***

NOTE:
These calculations are based on the assumption that DNA sequences are entirely random and that every nucleotide position has an equal probability of being A, T, C, or G. This is not always the case. In most genomes, certain sequences and regions can be repeated multiple times and a particular nucleotide does not always have an equal probability of being A, T, C, or G. In addition, some Cas enzymes have been shown to cut in slightly non-homologous regions. Various online tools, such as CRISPR design software and NCBI's Basic Local Alignment Search Tool (BLAST), help researchers incorporate these variabilities into their gRNA selection. Your class may also complete Appendix B and use BLAST to determine the specificity of your gRNAs based on sequenced genome data.

Post Lab Questions *(Answer after completing Module I)*

1. During the design process of Module I Part A, was finding a Cas9 PAM sites more or less of a challenge than you expected?

2. In Module I Part B, you determined probability of finding a sequence that exactly matched your first gRNA-Cas9 system (question 3, page 18) and you determined how many times you would expect to see this sequence in the *E. coli* genome (question 4, page 19). What would these numbers be for the four remaining gRNAs-Cas9 systems that you designed? Did you need to calculate these numbers for each individual gRNA, why or why not?

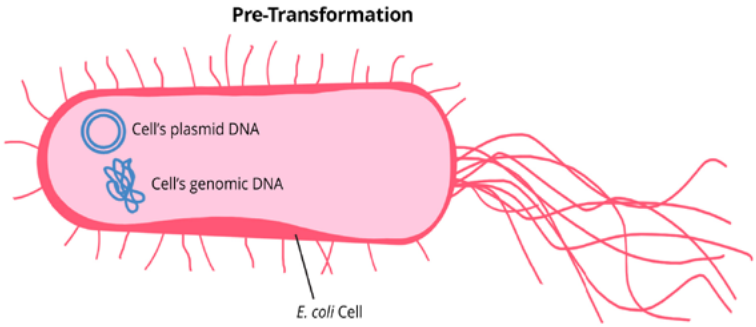
3. How many times would you expect to find a 20 bp gRNA with a Cas9 PAM site sequence in the human genome? (The size of the human genome is ~31 billion base pairs or 3.1×10^9 bp. Occurrence can be calculated by multiplying the genome by the sequence's probability). Brainstorm at least one reason why the chance of off target cut sites during an experiment might be bigger than this number suggests.

4. How does the accuracy of CRISPR-Cas compare to that of restriction enzyme digestion such as *Eco*RI? Provide a mathematical example using your calculations in Module I Part B and the fact that *Eco*RI's recognizes the specific palindromic DNA sequence 5'-GAATTC-3'.

Module II Experiment Summary

THE CELL

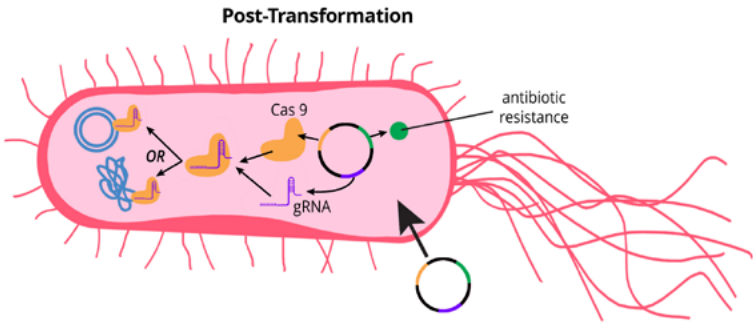
- **LyphoCells™ *E. coli* Purple:** The host bacteria have been genetically engineered to produce a purple pigment protein in the presence of IPTG. The genome has been modified to contain the gene for T7 polymerase under the control of the lac operon. The bacteria have also been transformed with a plasmid containing the Purple Chromoprotein under the control of the T7 promoter. When IPTG is added it derepresses (activates) the lac operon which allows the cell to synthesize T7 polymerase. This polymerase binds to the plasmid and turns on production of the purple protein.



THE PLASMIDS

- **pCas9-PurpleGuide:**
  This plasmid contains the Cas9 gene and a gRNA that targets the purple chromoprotein gene. It also contains a chloramphenicol resistance gene.

- **pCas9-ControlGuide:**
  This plasmid contains the Cas9 gene and a non-targeting guide RNA with a random sequence. This sequence was carefully checked against the *E. coli* purple genome and confirmed to be absent, ensuring that it won't target any genomic sites during the experiment. The plasmid also contains a chloramphenicol resistance gene.



THE PLATES

This experiment uses three additives to the plates:

- **IPTG (Isopropyl β-D-1-thiogalactopyranoside):**
  This compound induces the expression of genes controlled by the lac operon. It mimics the natural inducer of the lac operon, lactose, but unlike lactose, it is not metabolized by the cell, making it a more controlled and reliable inducer for gene expression.

- **Chloramphenicol:**
  A popular antibiotic and selective agent in bacterial culture media. Effective against a range of bacteria by inhibiting their protein synthesis.

- **Ampicillin :**
  A broad-spectrum antibiotic belonging to the penicillin group that works by inhibiting the synthesis of bacterial cell walls.

Module II: Pre-Lab Questions

(Answer after reading Module II's instructions but BEFORE performing Module II)

1. List and describe the two key elements involved in the genetic engineering process for each of your CRISPR experiments:

   - Experiment 1 (pCas9-PurpleGuide)

   - Experiment 2 (pCas9-ControlGuide)

2. How are these elements introduced to *E. coli* cells?

3. Why do you think this experiment adds chloramphenicol to the plates and includes a chloramphenicol resistance gene in both the Purple Guide plasmid and the Control Guide plasmid?

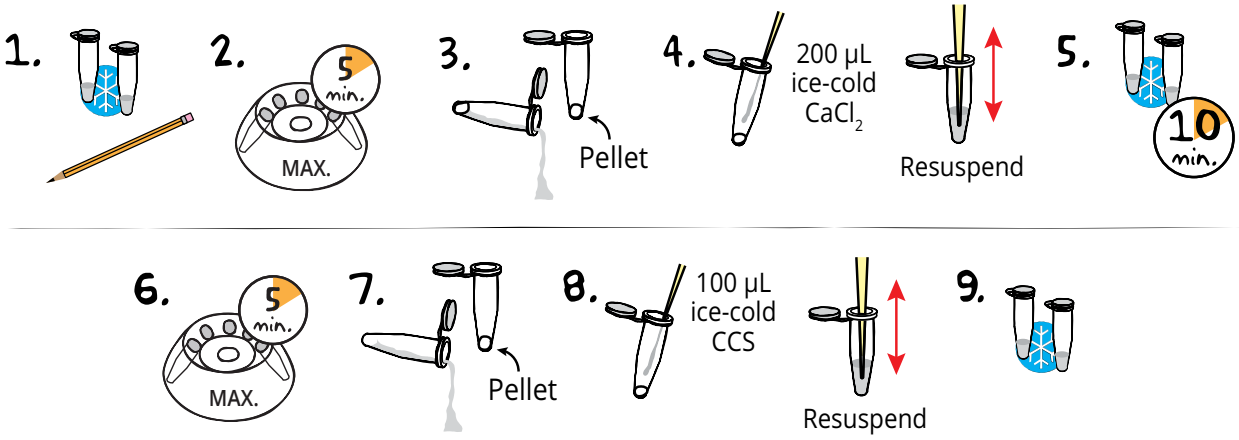
4. Which DNA repair mechanism (NHEJ or HDR) is being used in your CRISPR experiments? In a successful CRISPR experiment, how does this mechanism change the target gene?

5. Briefly explain how colony color can be used as evidence of the purple chromoprotein gene's state (functional or non-functional). In which experiment do you expect to observe a color change from purple to white?

6. Compare and contrast your two CRISPR experiments (cell strain, plasmid, procedure, plate additives etc.). How are they similar, and how are they different? What does each experiment demonstrate?

Module II-A: Preparation of Competent Cells

PREPARATION OF COMPETENT CELLS

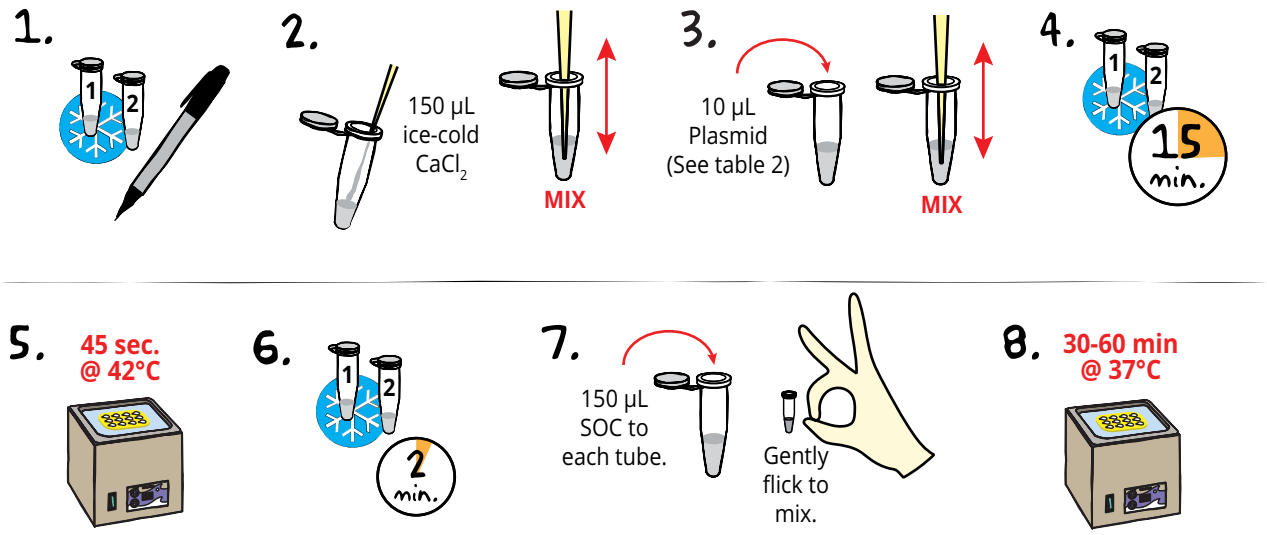


NOTE: Keep tubes on ice as much as possible during this module.

1. **OBTAIN** two 1.5 mL tubes of *E. coli* starter culture. **LABEL** the tubes with your initials or group number.
2. **CENTRIFUGE** the tubes at maximum speed for 5 minutes to pellet the cells.
3. Carefully **POUR** off the supernatant. DO NOT DISTURB THE CELL PELLETS!
4. **ADD** 200 µL ice cold $CaCl_2$ solution to each tube. Next, gently **RESUSPEND** the cells by slowly pipetting up and down several times. *NOTE: It is important that the cells are fully resuspended. Continue to gently pipette until no clumps are seen in the $CaCl_2$ solution.*
5. **INCUBATE** tubes on ice for 10 minutes.
6. **CENTRIFUGE** the tubes at maximum speed for 5 minutes to pellet the cells.
7. Carefully **POUR** off supernatant. DO NOT DISTURB THE CELL PELLETS! *NOTE: At this point, the cells are fragile. Keep the cells on ice and pipette slowly and gently in steps 9 and 10.*
8. Slowly **ADD** 100 µL ice cold Competent Cell Solution (CCS) to both tubes. Next, gently **RESUSPEND** the cells in the ice cold solution by slowly pipetting up and down several times.
9. Immediately **PLACE** the tubes on ice and proceed to Module II-B: Transformation.

OPTIONAL STOPPING POINT: The competent cells can be stored for up to 48 hours in the freezer after they have been resuspended in competent cell solution.

Module II-B: Transformation



NOTE: Keep tubes on ice as much as possible during this module.

1. **COLLECT** your two tubes of competent cells. **LABEL** them "1" and "2". **ENSURE** that the cells are kept on ice at all times.
2. **ADD** 150 µL ice cold $CaCl_2$ solution to each tube and **MIX** by gently pipetting up and down several times.
3. Using a new pipette tip for each tube, **ADD** 10 µL of the appropriate plasmids to each tube (see Table 2). **MIX** by gently pipetting up and down several times.
4. **INCUBATE** tubes on ice for 15 minutes.
5. *Quickly* **PLACE** the transformation tubes in a 42°C water bath for exactly 45 seconds.
6. *Immediately* **RETURN** the tubes to the ice bucket and **INCUBATE** for 2 minutes.
7. Using a new pipette tip for each tube, **ADD** 150 µL of SOC solution to each tube. **MIX** by gently flicking each tube.
8. **PLACE** tubes in a 37°C water bath and **INCUBATE** for 30-60 minutes to allow for recovery.

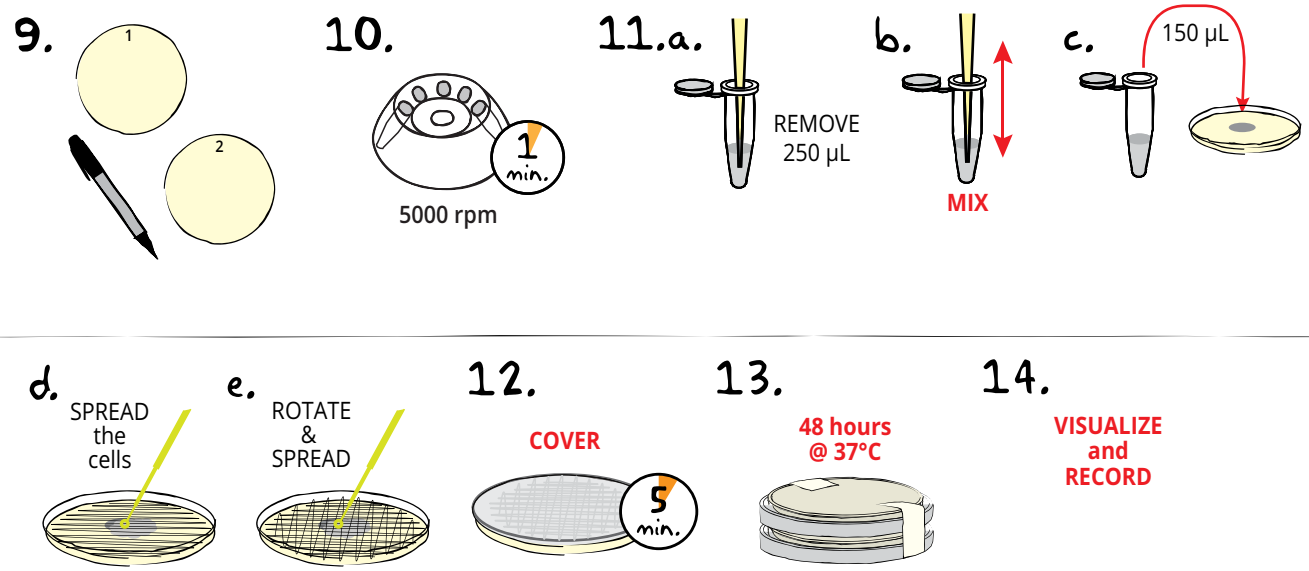
continued

TABLE 2: Experiment Overview

Experiment	Bacteria Strain	Plasmid	Plate
1	*E. coli* Purple	pCas9-PurpleGuide "PG"	Amp/IPTG/Chloramphenicol
2	*E. coli* Purple	pCas9-ControlGuide "CG"	Amp/IPTG/Chloramphenicol

Module II-B: Transformation, continued

TRANSFORMATION, continued



9. While cells are recovering, **COLLECT** and **LABEL** the bottom of two agar plates as indicated below.
   NOTE: Keep writing small and along the edge so you can easily see your colonies at the end.

   - 1 or "PG" for pCas9-PurpleGuide
   - 2 or "CG" for pCas9-ControlGuide

10. Following recovery, **SPIN** the tubes at 5000 rpm for 1 minute.
11. **PLATE** your two transformed cell lines by performing the following five steps one tube at a time. Use a new sterile pipet tip and loop for each tube/plate.
    (a) **REMOVE** 250 µL of the media. Work carefully during this step to making sure the cell pellet stays in place.
    (b) Gently **RESUSPEND** the pellet in the remaining media by pipetting up and down several times until no clumps are visible.
    (c) **COLLECT** 150 µL of the resuspension and **PIPET** into the center of the appropriate agar plate.
    (d) Use a loop to **SPREAD** the cells evenly and thoroughly over the entire surface.
    (e) **TURN** the plate 90° and thoroughly **SPREAD** again.
12. **COVER** the plates with lids and **INCUBATE** at room temperature for 5 minutes to allow the liquid to be fully absorbed.
13. **STACK** the plates on top of one another and **TAPE** them together. **INVERT** plates (agar side on top) and **PLACE** in a 37°C bacterial incubation oven. **INCUBATE** for 48 hours. If you do not have an incubator, colonies will form at room temperature in approximately 48-72 hours, but an incubator is HIGHLY recommended.
14. **VISUALIZE** the transformation and control plates and **RECORD** the number of colonies on the plate, the color(s) of the colonies (such as dark purple, light purple, white), and the number of colonies of each color. If the colors are faint, the plates can be left in the refrigerator (4°C) for 24-48 hours to for further color development.

Module II: Post Lab Questions *(Answer AFTER completing Module II)*

1. Describe the results of each experiment. Do they match your pre-lab predictions?

2. Which experiment shows evidence of CRISPR occurring? Describe what happened in this experiment to the targeted DNA.

3. Knockout experiments are often used to study gene function by observing the effects of gene disruption. In which experiment did you observe a gene knockout? What did it tell you about the purple chromoprotein gene in *E.coli* purple cells?

4. The central dogma of molecular biology states that genetic information flows from DNA to RNA to protein. For experiment 1 and 2, describe what happens to the DNA, RNA, and protein following the attempted knock-out of the chromoprotein gene by CRISPR.

5. In CRISPR experiments, gRNAs are used and the result is a modified gene in the genomic DNA. Why does this not violate the central dogma?

6. Biological experiments are not 100% efficient. For example:

   - CRISPR/Cas9 can sometimes bind to and cut DNA sequences that are similar, but not identical, to the intended target. These off-target cuts can reduce the efficiency of the desired gene editing.
   - The efficiency of transformation depends on the competency of the cells, the nature of the plasmid, and laboratory technique. Even under optimal conditions, only a fraction of cells in a bacterial population are able to take up foreign DNA.
   - Even if a cell successfully takes up the plasmid, it may lose the plasmid during subsequent cell divisions, especially if the plasmid does not confer a strong selective advantage.

   Look at your experimental results. Do you observe any signs of inefficiency?

Instructor's Guide

ADVANCED PREPARATION

This CRISPR experiment requires advance preparation by the instructor before the day of the lab. Some of the reagents can be prepared and stored ahead of time, as indicated by the table, below. Please read the instructions carefully while planning your lesson.

Instructors will create SOC agar plates, let them solidify and dry overnight, and then store for up to 7 days. In addition, the transformation reagents can be dispensed and stored at 4°C for one day if needed. If time is limited, instructors can prepare competent cells (Module II A) as part of the pre-lab rather than as a student activity.

ADVANCE PREPARATION:		
What to do:	**Time Required:**	**When?**
Prepare SOC Plates	60 minutes	2-7 days before experiment.
Dispense plasmid DNA, $CaCl_2$, CCS solution, & SOC solution.	30 minutes	One day to 30 min. before performing the experiment.
Prepare *E.coli* source plates	20 minutes to streak 18-48 hours to incubate	18-22 hours before the experiment for best results. Can be prepared 24-48 hrs before at room temp.
DAY OF THE EXPERIMENT:		
What to do:	**Time Required:**	**When?**
Prepare *E.coli* starter culture	70-90 minutes	70-90 min. before the experiment for best results. Can be prepared up to 3 days before the experiment.
Prepare Competent Cells	30 minutes	30 min. before the transformation for best results. Can be prepared up to 2 days before transformation.
Equilibrate water baths at 37°C and 42°C; incubator at 37°C	10 minutes	1-2 hours before performing the experiment.
Perform laboratory experiment	1-1.5 hrs plus add 30 min. if students prepare competent cells.	The class period.
Incubate cells.	48 hours at 37°C or 48-72 hours at room temp.	Overnight after the class period.
RESULTS AND CLEAN UP:		
What to do:	**Time Required:**	**When?**
Students observe the results of their experiment	30-45 minutes	The following class period.
Discard any contaminated materials	45 minutes - overnight	After the students have analyzed their results.

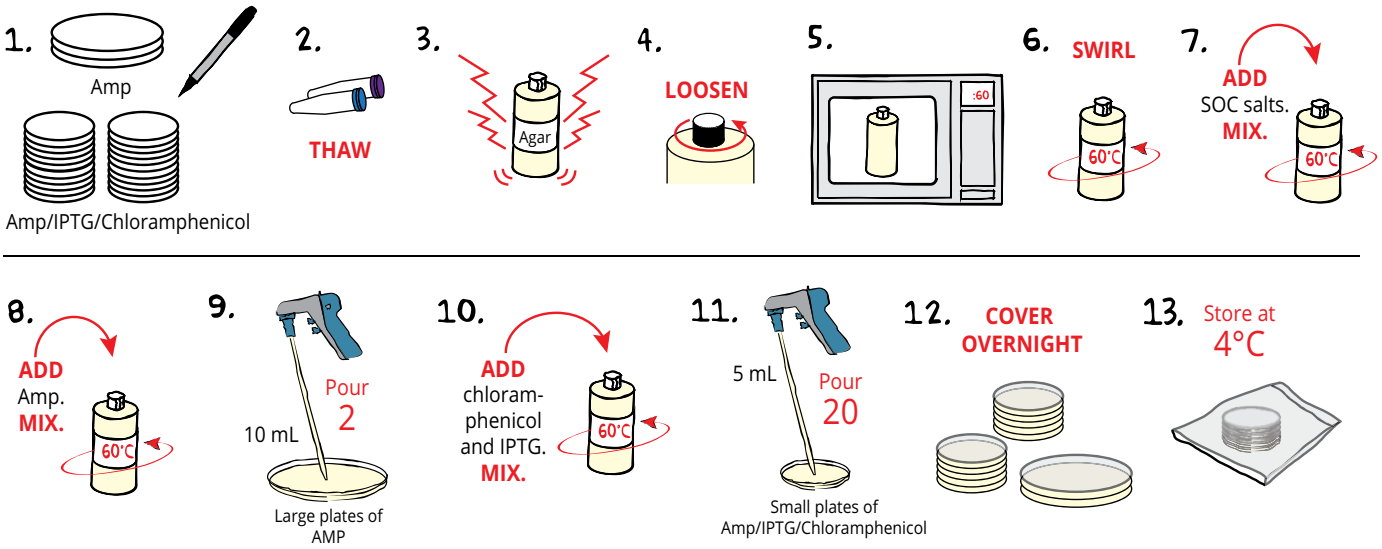
IMPORTANT READ ME

Transformation experiments contain antibiotics which are used for the selection of transformed bacteria. Students who have allergies to antibiotics such as penicillin, ampicillin, kanamycin or tetracycline should not participate in this experiment.

Pre-Lab Preparations

POURING SOC-AGAR PLATES

You will be preparing plates for use during Module II of the experiment. For the source plate (*E. coli* purple) you will prepare two large AMP plates. For the student experiment, you will prepare 20 small amp/IPTG/Chloramphenicol plates.



1. **LABEL** 2 large plates "Amp" and 20 small plates "Amp/IPTG/Chloramphenicol". Keep writing small and along the edge so student's can easily see the final colonies.
2. **PLACE** the tubes of IPTG and Chloramphenicol on the counter to thaw. Before dispensing, **TAP** both plasmid tubes until the samples are at the tapered bottom of the tubes.
3. **BREAK** solid ReadyPour™ SOC Agar into small chunks by vigorously squeezing and shaking the plastic bottle.
4. **LOOSEN**, but DO NOT REMOVE, the cap on the ReadyPour™ SOC Agar bottle. This allows the steam to vent during heating. ***CAUTION: Failure to loosen the cap prior to heating may cause the bottle to break or explode.***
5. **MICROWAVE** the ReadyPour™ SOC Agar on high for 60 seconds to melt the agar. Carefully **REMOVE** the bottle from the microwave and **MIX** by swirling the bottle. Continue to **HEAT** the solution in 30-second intervals until the agar is completely dissolved (the amber-colored solution should be clear and free of small particles).
6. **SWIRL** to promote even dissipation of heat and to ensure that temperatures are close to 60°C.
7. **ADD** the entire amount of SOC Additive to the molten ReadyPour™. Swirl to **MIX**.
8. **ADD** the entire amount of the Ampicillin to the ReadyPour™ SOC Agar and **MIX**.
9. **POUR** 10 mL of the agar solution into the two large plates labeled "Amp". Set these aside to solidify.
10. **ADD** the entire volume of chloramphenicol and IPTG to the agar solution and **MIX**.
11. **POUR** 5 mL each into the twenty small plates labeled "Amp/IPTG/Chloramphenicol".
12. **COVER** and **WAIT** for the agar plates to solidify. For optimal results, leave plates at room temperature overnight.
13. **STORE** plates in the refrigerator (4°C) until needed. Plates should be inverted and placed in a sealable plastic bag to ensure that they do not dry out.

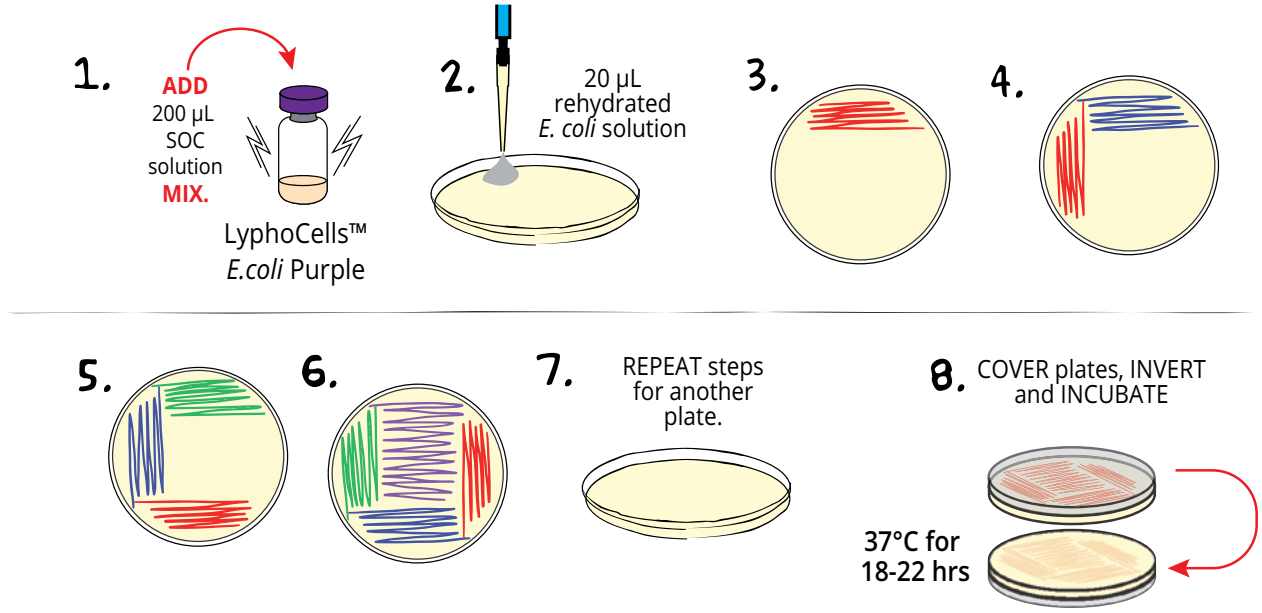
REMINDER:
Only add reagents to cooled agar (60°C).

NOTE:
If plates are prepared more than one day before use, they should be left on the bench overnight to dry. The following day, store plates inverted in a plastic bag in the refrigerator (4°C). Remove the plates from the refrigerator and warm in a 37°C incubator for 30 minutes before use.

Pre-Lab Preparations

PREPARATION OF *E. coli* SOURCE PLATES

For best results, the *E. coli* source plates should be streaked 18-22 hours before the preparation of competent cells. Preparing the source plates more than 24 hours before may compromise the success of the transformation experiment.



1. **REMOVE** 200 µL SOC liquid medium to the LyphoCells™ *E. coli* Purple vial. **SAVE** the remaining SOC @ 4°C until needed. Gently **SHAKE** the vilal to resuspend the bacteria.
2. **PIPETTE** 20 µL of rehydrated *E. coli* to the edge of one of the large AMP petri plates.
3. **STREAK** the loop back and forth through the bacteria to make a primary streak at the top of the plate. Try not to gouge the loop into the medium.
4. **ROTATE** the plate 90°. **STREAK** the loop through the primary streak once, then zig-zag across a clean part of the agar several times to create a secondary streak.
5. **ROTATE** the plate. **STREAK** the loop through the secondary streak once and then across a clean part of the agar several times.
6. **ROTATE** the plate once more. **STREAK** the loop through the third streak and then zig-zag across the remaining clean agar. This should produce isolated colonies.
7. **REPEAT** steps 2 through 6 for the second source plate using a new loop.
8 **COVER** the two plates and **INCUBATE INVERTED** at 37°C for 18-22 hours. If you do not have an incubator, colonies will form at room temperature in approximately 24-48 hours, although transformation efficiency will decrease.

*One source plate will be used to prepare the *E. coli* starter cultures. The second source plate can be saved as a backup if needed.

Pre-Lab Preparations

PREPARATION OF WATER BATHS AND INCUBATOR

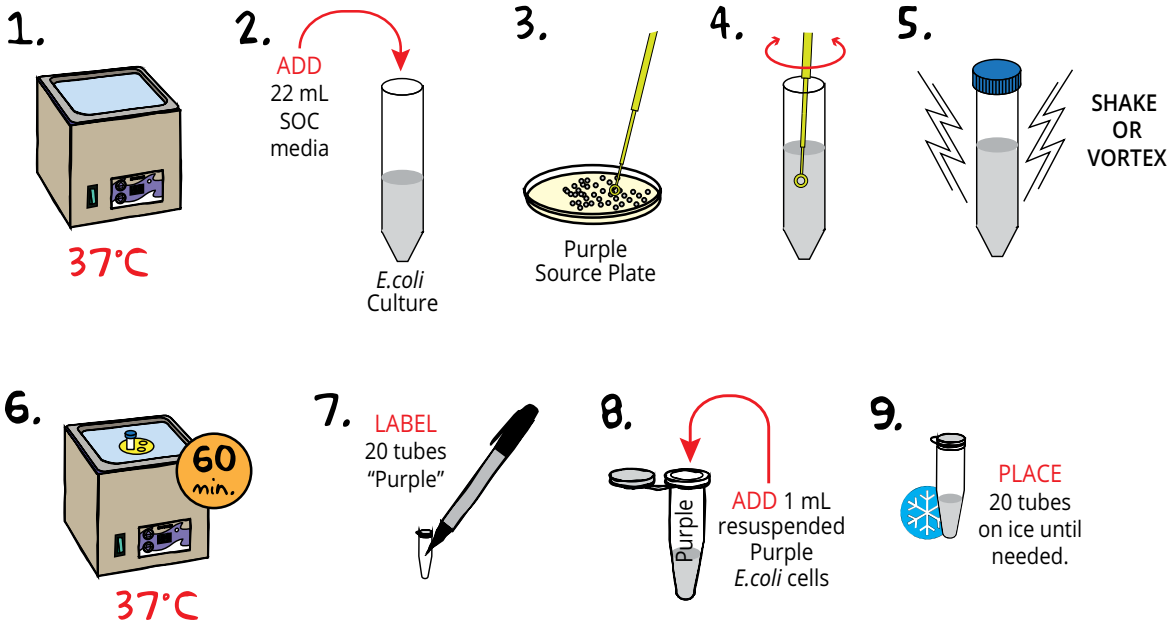
Students will need two water baths for the CRISPR procedure. Equilibrate the water baths at 37° C and 42°C ahead of the experiment to ensure the temperatures are accurate. Ensure that water baths fit all 40 tubes before the experiment.

Quick access to 42°C water baths is particularly important. An extra 42°C water baths can prevent bottlenecks at Module II step 5. Alternatively, consider slightly staggering groups during Module II steps 4, 5 and 6.

In addition, set a bacterial incubator (if using) to 37°C for overnight incubation of the CRISPR plates on the day of the experiment.

DAY OF THE LAB: Preparation of *E. coli* Starter Cultures

For best results, the *E. coli* starter should be prepared 70-90 minutes before the experiment is performed.



1. **PREPARE** a 37°C water bath.
2. **ADD** 22 mL of SOC liquid medium to a 50 mL conical tube and **LABEL** it "*E. coli* Culture".
3. **SWIPE** an inoculation loop through a dense section of the bacterial culture from one of your purple source plate. You want to collect a clump of bacteria that fills the loop. The collected clump should be approximately the size of a match head.
4. **RESUSPEND** the bacteria in the "*E. coli* Culture" tube by twisting the loop back and forth until all bacteria have been removed from the loop.
5. **SHAKE** or vortex the tube briefly to ensure that the bacteria are completely resuspended.
6. **INCUBATE** the tube for 60 minutes in a 37°C water bath.
7. **LABEL** 20 snap-top microcentrifuge tubes as "*E. coli*" or "Starter culture ".
8. **ALIQUOT** 1 mL of resuspended cells in the "*E. coli* Culture" into each tube.
9. **PLACE** the tubes on ice until they are needed for the experiment (Module II: Preparation of Competent Cells).

Pre-Lab Preparations

Prepare Plasmid DNA

1. **PLACE** the tubes of pCas9-PurpleGuide and pCas9-ControlGuide on ice to thaw.
2. **LABEL** 10 microcentrifuge tubes "PG" and 10 microcentrifuge tubes "CG".
3. Before dispensing, **TAP** both plasmid tubes until the samples are at the tapered bottom of the tubes.
4. Using an adjustable volume micropipette, **DISPENSE** 13 µL of the pCas9-PurpleGuide plasmids to each of the microcentrifuge tubes labeled "PG". **CAP** the tubes and **PLACE** them on ice.
5. Using a fresh pipette tip, **DISPENSE** 13 µL of the pCas9-ControlGuide plasmids to each of the microcentrifuge tubes labeled "CG". **CAP** the tubes and **PLACE** them on ice.

Additional Pre-Lab Preparations (Day of Lab)

6. **PREPARE** ice or ice-water baths for each group. Small ice cubes will help to rapidly cool the bacteria after the heat shock.
7. **DISPENSE** 750 µL $CaCl_2$ into ten microcentrifuge tubes, **LABEL** "$CaCl_2$" and **PLACE** tubes on ice. ***NOTE: This $CaCl_2$ solution will be used in both Modules II-A and II-B. Make sure students keep the solution between modules.***
8. **DISPENSE** 220 µL Competent Cell Solution into ten microcentrifuge tubes, **LABEL** "CCS" and **PLACE** tubes on ice.
9. **DISPENSE** 330 µL SOC liquid medium into ten microcentrifuge tubes, **LABEL** "SOC" and **PLACE** tubes on ice.
10. **EQUILIBRATE** water baths at 37°C and 42°C; **SET** the incubator at 37°C. **CONFIRM** the temperature with a thermometer

NOTE: If prepared ahead of time, the plasmids and $CaCl_2$ aliquots can be stored at 4°C for up to 24 hours. Always provide plasmid DNA and $CaCl_2$ on ice to assist the heat shock procedure.

FOR MODULE I A&B
Each group/student requires:
- Pen/Pencil
- Highlighter

FOR MODULE II-A
Each group requires:
- 2 tubes *E. coli* purple starter culture
- Ice-cold $CaCl_2$ solution (750 µL)
- Ice-cold Competent Cell Solution (CCS) solution (220 µL)
- Ice and Ice Bucket
- Pipet and Pipet Tips
- Sharpie
- Gloves

Classroom equipment:
- Centrifuge

FOR MODULE II-B
Each group requires:
- 2 tubes competent *E. coli* purple cells
- Ice-cold $CaCl_2$ solution (350 µL)
- Tube labeled "PG" (13 µL)
- Tube labeled "CG" (13 µL)
- SOC solution (330 µL)
- 2 A/C/I Plates
- Pipette and Pipette Tips (0-200 µL min, 0-10 µL and 0-1000 µL also useful)
- 2 loops
- Pipet and Pipet Tips
- Sharpie
- Lab tape
- Gloves

Classroom equipment:
- Water baths (37°C and 42°C)
- Centrifuge
- Optional 37°C incubator oven

GUIDE TO MODULE II RESULTS AND ANALYSIS

- Encourage students to take a picture of their plate results to put in their lab book. In addition to recording the number of colonies on the plate, students should record color(s) of the colonies (such as dark purple, light purple, white), and the number of colonies of each color. Remind them to record relevant experimental facts such as the bacteria, plasmid, and gRNA target.
- Students can calculate the gene editing efficiency of both experiments by calculating the percentage of light purple or white (CRISPR) colonies over the total number of colonies on a plate.
- The CRISPR-Cas9 gene editing system in this kit is highly efficient, but it may not be 100% effective in all cases. Some students may observe purple colonies on their Experiment 1 plates, which result from bacteria with purple chromoprotein genes that were not modified. In other cases, there may be a contrast between Experiment 1 and Experiment 2, with lighter versus darker purple colonies. This may indicate that CRISPR successfully knocked down chromoprotein expression but did not fully knock it out.

Module I - Results

MODULE IA: DESIGNING gRNA

There are 33 potential PAM sites in the provided sequence. The answers given below are examples. Page 34 provides a full list of possible PAM sites.

1. 5′ – ATGACCATGATTACGGATTCACTGGCCGTCGTTTTACAACGTCGTGACTG
TACTGGTACTAATGCCTAAGTGACCGGCAGCAAAATGTTGCAGCACTGAC

GGAAAACCCTGGCGTTACCCAACTTAATCGCCTTGCAGCACATCCCCCTT
CCTTTTGGGACCGCAATGGGTTGAATTAGCGGAACGTCGTGTAGGGGGAA

TCGCCAGCTGGCGTAATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAA
AGCGGTCGACCGCATTATCGCTTCTCCGGGCGTGGCTAGCGGGAAGGGAA

CAGTTGCGCAGCCTGAATGGCGAATGGCGCTTTGCCTGGTTTCCGGCACC – 3′
GTCAACGCGTCGGACTTACCGCTTACCGCGAAACGGACCAAAGGCCGTGG – 5′

2. 5′ – ATGACCATGATTACGGATTCACTGGCCGTCGTTTTACAACGTCGTGACTG
TACTGGTACTAATGCCTAAGTGACCGGCAGCAAAATGTTGCAGCACTGAC

GGAAAACCCTGGCGTTACCCAACTTAATCGCCTTGCAGCACATCCCCCTT
CCTTTTGGGACCGCAATGGGTTGAATTAGCGGAACGTCGTGTAGGGGGAA

TCGCCAGCTGGCGTAATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAA
AGCGGTCGACCGCATTATCGCTTCTCCGGGCGTGGCTAGCGGGAAGGGAA

CAGTTGCGCAGCCTGAATGGCGAATGGCGCTTTGCCTGGTTTCCGGCACC – 3′
GTCAACGCGTCGGACTTACCGCTTACCGCGAAACGGACCAAAGGCCGTGG – 5′

Module I - Results, continued

MODULE IA: DESIGNING gRNA, continued

3.

GCAGCACTGACCCTTTTGGG
CGUCGUGACUGGGAAAACCC

TTCGCCAGCTGGCGTAATAG
AAGCGGUCGACCGCAUUAUC

GTCGACCGCATTATCGCTTC
CAGCUGGCGUAAUAGCGAAG

GAATGGCGAATGGCGCTTTG
CUUACCGCUUACCGCGAAAC

TACCGCTTACCGCGAAACGG
AUGGCGAAUGGCGCUUUGCC

4.

TABLE 1

gRNA Name	Guide Sequence (RNA)	PAM Sequence (DNA)
gRNA 1	CGUCGUGACUGGGAAAACCC	TGG
gRNA 2	AAGCGGUCGACCGCAUUAUC	AGG
gRNA 3	CAGCUGGCGUAAUAGCGAAG	AGG
gRNA 4	CUUACCGCUUACCGCGAAAC	AGG
gRNA 5	AUGGCGAAUGGCGCUUUGCC	TGG

MODULE I-B: ESTIMATING SPECIFICITY

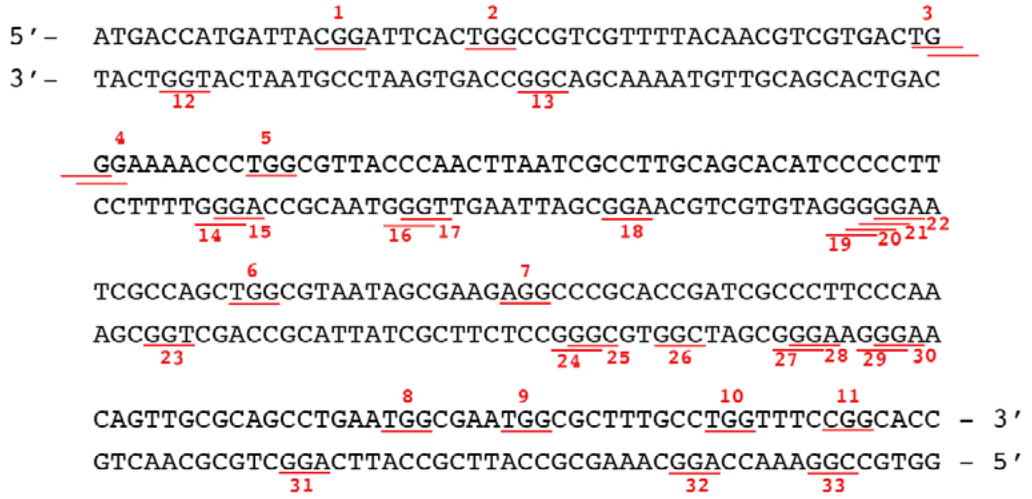
1. 1/4
   (The probability of getting a certain nucleotide is 1/4 because DNA and RNA are composed of four different nucleotides.)

2. 1/4*1/4*1/4*1/4*1/4*1/4*1/4*1/4*1/4*1/4*1/4*1/4*1/4*1/4*1/4*1/4*1/4*1/4*1/4*1/4*1/4*1/4 or $(1/4)^{20}$
   =1/1,099,511,600,000
   =9.0949472e-13 (0.0000000000090949472)
   (There are 20 nucleotides in the gRNA sequence and the probability for having a specific nucleotide is 1/4.)

3. 1/1,099,511,600,000*1*1/4*1/4 = 5.6843419e-14 (0.000000000000056842419)
   (There are 3 nucleotides in the PAM sequence but one of these is 'N' which can be any of the 4 nucleotides. The chance of being any of the 4 nucleotides is 100% or 1.)

4. 5.6843419e-14*5,000,000 = 2.84217094e-7 (0.000000284217094)

Module I - Results, continued



gRNA #	Target	PAM Site	Strand	Starting Nucleotide of PAM site (Row, Position)
gRNA 1	AUGACCAUGAUUA (incomplete)	CGG	5' to 3' (Top)	1, 14
gRNA 2	GACCAUGAUUACGGAUUCAC	TGG	5' to 3' (Top)	1, 23
gRNA 3	UCGUUUUACAACGUCGUGAC	TGG	5' to 3' (Top)	1, 49
gRNA 4	CGUUUUACAACGUCGUGACU	GGG	5' to 3' (Top)	1, 50
gRNA 5	CGUCGUGACUGGGAAAACCC	TGG	5' to 3' (Top)	2, 10
gRNA 6	CACAUCCCCCUUUCGCCAGC	TGG	5' to 3' (Top)	3, 9
gRNA 7	CAGCUGGCGUAAUAGCGAAG	AGG	5' to 3' (Top)	3, 25
gRNA 8	CAACAGUUGCGCAGCCUGAA	TGG	5' to 3' (Top)	4, 18
gRNA 9	UGCGCAGCCUGAAUGGCGAA	TGG	5' to 3' (Top)	4, 25
gRNA 10	AUGGCGAAUGGCGCUUUGCC	TGG	5' to 3' (Top)	4, 37
gRNA 11	AUGGCGCUUUGCCUGGUUUC	CGG	5' to 3' (Top)	4, 44
gRNA 12	GGCCAGUGAAUCCGUAAUCA	TGG	3' to 5' (Bottom)	1, 5
gRNA 13	GUCACGACGUUGUAAAACGA	CGG	3' to 5' (Bottom)	1, 26
gRNA 14	GAUUAAGUUGGGUAACGCCA	GGG	3' to 5' (Bottom)	2, 7
gRNA 15	CGAUUAAGUUGGGUAACGCC	AGG	3' to 5' (Bottom)	2, 8
gRNA 16	UGCUGCAAGGCGAUUAAGUU	GGG	3' to 5' (Bottom)	2, 18
gRNA 17	GUGCUGCAAGGCGAUUAAGU	TGG	3' to 5' (Bottom)	2, 19
gRNA 18	CGAAAGGGGGAUGUGCUGCA	AGG	3' to 5' (Bottom)	2, 31
gRNA 19	UUACGCCAGCUGGCGAAAGG	GGG	3' to 5' (Bottom)	2, 44
gRNA 20	AUUACGCCAGCUGGCGAAAG	GGG	3' to 5' (Bottom)	2, 45
gRNA 21	UAUUACGCCAGCUGGCGAAA	GGG	3' to 5' (Bottom)	2, 46
gRNA 22	CUAUUACGCCAGCUGGCGAA	AGG	3' to 5' (Bottom)	2,47
gRNA 23	CUCUUCGCUAUUACGCCAGC	TGG	3' to 5' (Bottom)	3, 4
gRNA 24	AAGGGAAGGGCGAUCGGUGC	GGG	3' to 5' (Bottom)	3, 28
gRNA 25	GAAGGGAAGGGCGAUCGGUG	CGG	3' to 5' (Bottom)	3, 29
gRNA 26	CAACUGAAGGGAAGGGCGAU	CGG	3' to 5' (Bottom)	3, 34
gRNA 27	GGCUGCGCAACUGAAGGGAA	GGG	3' to 5' (Bottom)	3, 41
gRNA 28	AGGCUGCGCAACUGAAGGGA	AGG	3' to 5' (Bottom)	3, 42
gRNA 29	AUUCAGGCUGCGCAACUGAA	GGG	3' to 5' (Bottom)	3, 46
gRNA 30	CAUUCAGGCUGCGCAACUGA	AGG	3' to 5' (Bottom)	3, 47
gRNA 31	CAAAGCGCCAUUCGCCAUUC	AGG	3' to 5' (Bottom)	4, 12
gRNA 32	GGUGCCGGAAACC (incomplete)	AGG	3' to 5' (Bottom)	4, 35
gRNA 33	GGUGC (incomplete)	CGG	3' to 5' (Bottom)	4, 43

Module II - Results

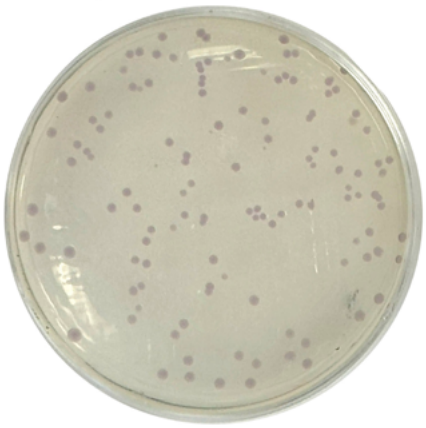
EXPERIMENT 1

Bacteria: *E. coli* Purple
Introduced Plasmid: pCas9-PurpleGuide
gRNA/CRISPR Target: purple chromoprotein gene
Final Colony Color: White
Did CRISPR occur? Yes.

The colony color change from purple to white suggests that *E. coli* Purple went from producing a purple pigment to no longer producing a purple pigment. This suggests that the CRISPR-Cas9 system was able to disrupt the purple chromoprotein gene.

Total number of colonies: 95
Number of dark purple colonies: 0
Number of light purple colonies: 0
Number of white colonies: 95

pCas9-PurpleGuide



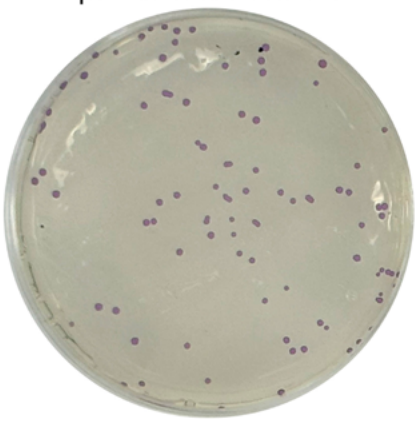
EXPERIMENT 2

Bacteria: *E. coli* Purple
Introduced Plasmid: pCas9-ControlGuide
gRNA/CRISPR Target: Random sequence not present in *E. coli* Purple
Final Colony Color: Purple
Did CRISPR occur? No.

The colony color did not change. This suggests that this CRISPR-Cas9 system was not able to disrupt the purple chromoprotein gene responsible for the bacteria's purple appearance. This result also confirms that the color change observed in experiment 1 was not solely due to the transformation process (such as an unexpected by-product of introducing the chloramphenicol resistance gene or another gene on the plasmid). Additionally, it demonstrates that Cas9 was unable to cut the purple chromoprotein gene without a specifically designed guide RNA.

Total number of colonies: 91
Number of dark purple colonies: 91
Number of light purple colonies: 0
Number of white colonies: 0

pCas9-ControlGuide



Please refer to the kit insert for the Answers to Study Questions

Appendices

A CRISPR Troubleshooting Guide

B BLAST Bioinformatics Activity

Safety Data Sheets can be found on our website: www.edvotek.com/safety-data-sheets

Appendix A
CRISPR Troubleshooting Guide

PROBLEM:	CAUSE:	ANSWER:
Poor cell growth on source plate	Incubation time too short	Continue to incubate source plate at 37ºC for a total of 18-22 hours.
	Incorrect incubation temperature	Use a thermometer to check incubator temperature. Adjust temp. to 37°C if necessary.
Satellite colonies seen on experiment plate	Incorrect concentration of antibiotics in plates	Ensure the correct concentration of antibiotic was added to plates - Make sure ReadyPour is cooled to 60°C before adding antibiotic.
	Antibiotic is degraded	Make sure ReadyPour is cooled to 60°C before adding antibiotic.
	Plates were incubated too long	Incubate the plates for 48 hours at 37ºC.
Colonies appeared smeary on experiment plate	Plates containing transformants were inverted too soon	Allow cells to fully absorb into the medium before inverting plates.
	Experimental plates too moist	After pouring plates, allow them dry overnight at room temp. Alternatively, warm plates at 37°C for 30 min. before plating cells
No colonies seen on experiment plates	Plasmid DNA not added to transformation mix	Ensure plasmid DNA was added to tubes.
		Make sure that pipets are used properly and are properly calibrated.
	Incorrect host cells used for experiment	Confirm that correct bacterial strain was used for transformation
	Cells were not properly heat shocked	Ensure that temp. was 42ºC & heat shock step took place for exactly 45 sec.
	Incorrect antibiotics	Be certain that the correct antibiotic was used.
	Cells not well resuspended in $CaCl_2$	Completely resuspend the cells in the $CaCl_2$, leaving no cell clumps (vortex or pipet up and down to fully resuspend cells). Cell suspension should be cloudy.
Low transformation efficiency (only a few colonies seen on experiment plates)	Too many injured or non-competent cells.	Fully but gently resuspend cells following centrifugation by slowly pipetting up and down. Ensure $CaCl_2$ and CCS are cold.
	Not enough cells used for experiment	Swipe through a dense section of the bacterial culture and use a full match sized loop. Incubate for full times. Keep on ice.
	Source plates were incubated for more than 20 hours	Important that source cells grow no longer than 22 hrs. Refrigerate plates after 22 hrs if necessary. Do not use source plates that have been incubated longer than 24 hours (refrigerated or not).
	Experimental plates too old	Prepare plate and use shortly after preparation
	Cells not well resuspended in $CaCl_2$	Completely resuspend the cells in the $CaCl_2$, leaving no cell clumps (vortex or pipet up and down to fully resuspend cells). Cell suspension should be cloudy.
	$CaCl_2$ solution not cold enough	Pre-chill $CaCl_2$ before adding cells to the $CaCl_2$
	Cell solution not cold enough	Extend incubation of cell suspension 10-15 minutes (should not exceed 30 min. total). This increases the transformation efficiency.
	Too much or too little plasmid DNA added to cell suspension	Ensure that correct volume of plasmid was added to the transformation tube. If using micropipets, make sure students practice using pipets.
	Cells were not properly heat shocked	Ensure that temperature was 42ºC and that heat shock step took place for no more than 45 seconds.
	Antibiotics were degraded prior to pouring plates	Make sure ReadyPour is cooled to 60°C before adding antibiotic.
	Incorrect concentration of antibiotics	Ensure that the correct concentration of antibiotic was used in plates.
Anything	---	Email us with your question at info@edvotek.com. Make sure to include the kit number and the guide version number (bottom of the page.)

Appendix B
BLAST Bioinformatics Activity

In this exercise you will use the Basic Local Alignment Search Tool (BLAST) to determine the specificity of the gRNAs you designed in Module IA. The BLAST tool finds regions of local similarity between a DNA sequence you specify and publicly available sequences in the GenBank data base. In BLAST terminology the user's input sequence is known as the query sequence, sequences in the database are known as target sequences, and sequences with similarities to the input sequence are hits, and regions of alignment between a query sequence and a hit sequence are matches.

COMPILE A LIST OF QUERY SEQUENCES

1. **SELECT** one gRNA from Table 1 (page 17.) **TRANSLATE** the RNA sequences back to DNA, then **COPY** the guide sequence four times* into a document or spreadsheet.

   _____

2. **ADD** the four possible PAM sites (AGG, CGG, GGG, and TGG) to the 3' end (right side) of the sequences you entered in step 1.

   _____

   _____

   _____

   _____

*In short sequence searchers BLAST cannot correctly process N (instead it's algorithm will ignore all subsequent nucleotides). Consequently you will need to perform four BLAST searches for your gRNA. One for each possible PAM site.

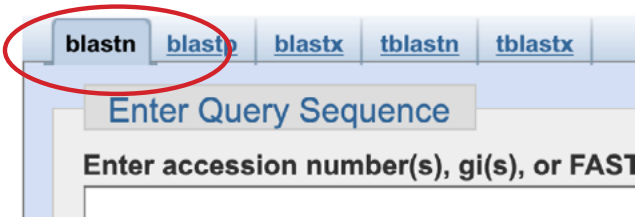
NOTE:
This activity has been designed using BLAST version 2.15.0. If you are having any problems email us at **info@edvotek.com**.

PERFORM A BLAST SEARCH

3. Type: **https://blast.ncbi.nlm.nih.gov/Blast.cgi** to go to the NCBI BLAST page.

4. On the BLAST home screen, click "Nucleotide BLAST".

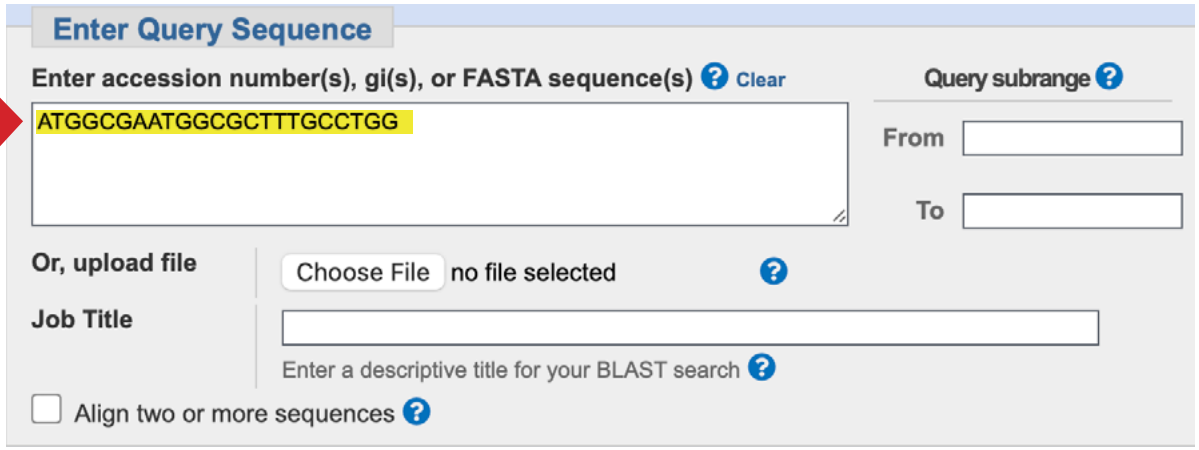
5. On the new screen, make sure the tab selected is "blastn" (IMAGE 2).



continued

Appendix B: BLAST Bioinformatics Activity, continued

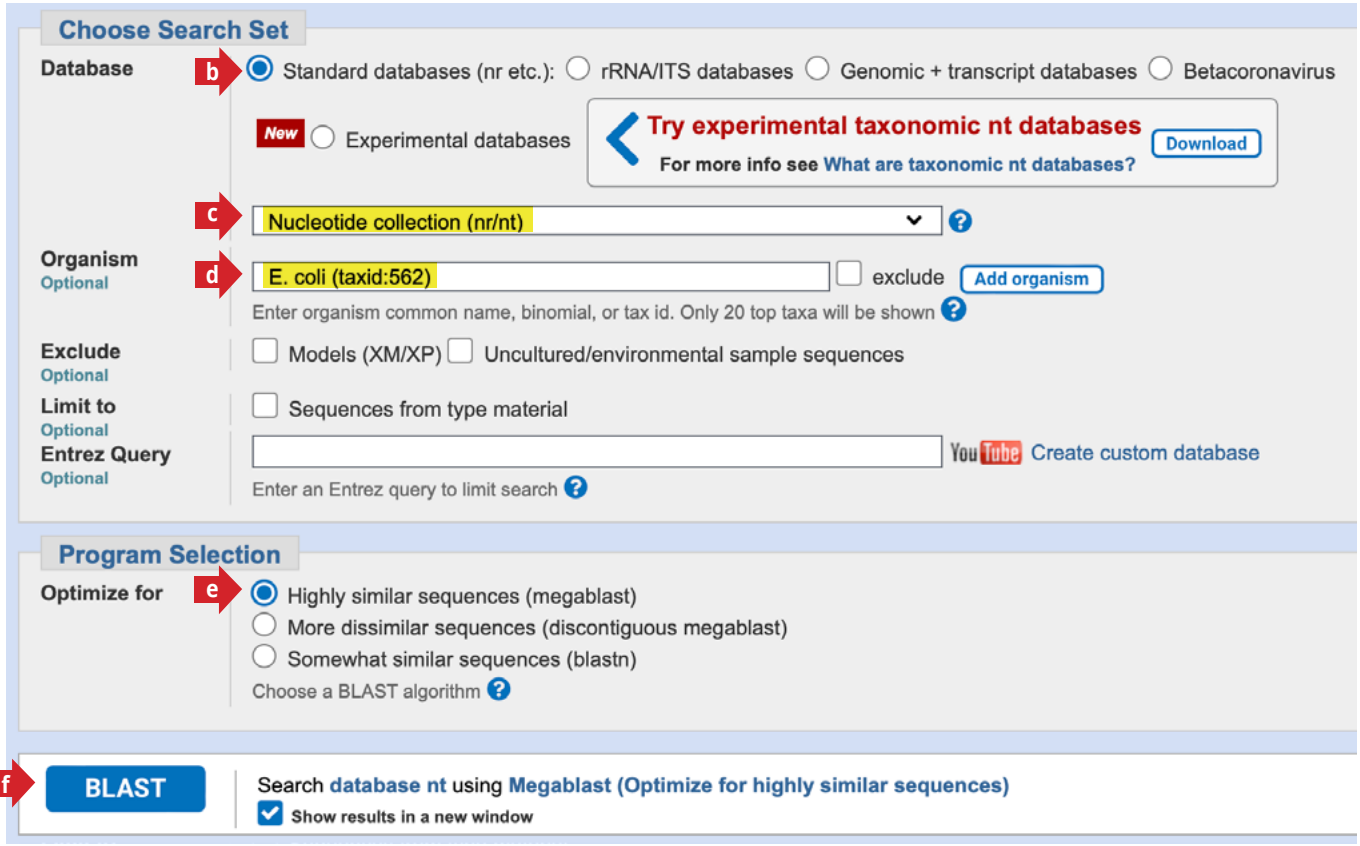
6. **ENTER** your first query sequence **(a)**.



7. Under "Choose Search Set", **SELECT** "Standard Database (nr etc)" **(b)** and **ENSURE** "Nucleotide collection (nr/nt)" is highlighted in the drop down menu **(c)**. In the "Organisms" box, begin writing "*E. coli*" and **SELECT** "*E. coli* (taxid: 562)" when it becomes available **(d)**. The remaining entries should be left bank.

8. Under "Program Selection" **SELECT** "Highly similar sequence (megablast)" **(e)**.

9. **CLICK** on the blue "BLAST" query box **(f)**.



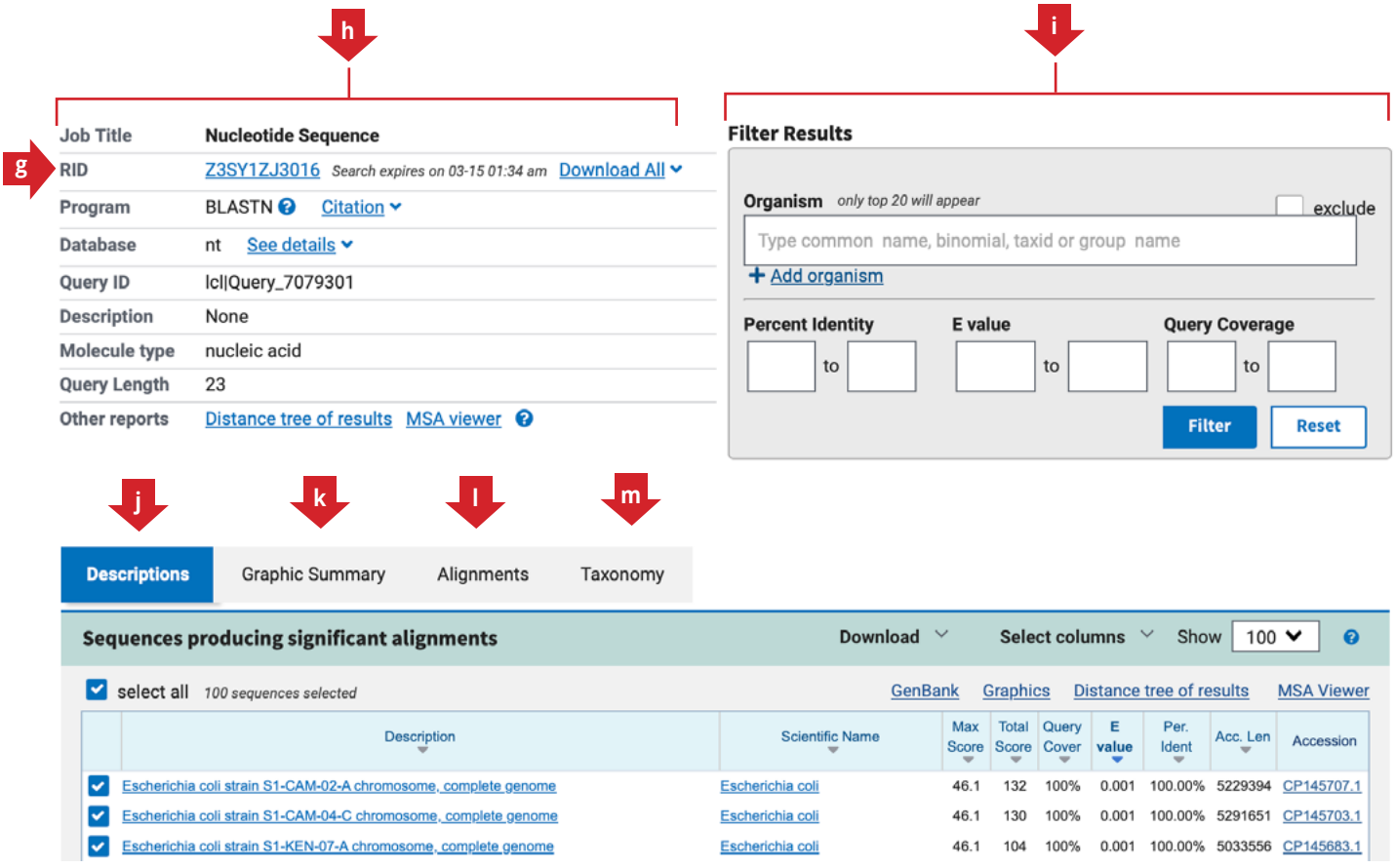
Appendix B: BLAST Bioinformatics Activity, continued

10. Once the "BLAST" query box has been clicked you will be assigned a Request ID# **(g)**. **RECORD** this number so you can check on your results at a later time. (Results are stored online for 48 hours.)

    REQUEST ID#_____

11. Briefly **EXAMINE** the BLASTN search report. The report includes:
    - **(h)** SEARCH SUMMARY report showing an overview of the BLASTN search parameters.
    - **(i)** FILTER RESULTS section where you can further specify the organism you want BLASTN to compare your sequence to.
    - **(j)** DESCRIPTIONS tab that shows all the sequences in the database with significant sequence homology to the sequence.
    - **(k)** GRAPHIC SUMMARY tab that shows the alignment of database matches to the query sequence. The color of the boxes correspond to the score of the alignment.
    - **(l)** ALIGNMENT tab that shows alignment blocks for each BLAST hit. Each alignment block begins with a summary that includes information about the match including the Max score and expected value.
    - **(m)** TAXONOMY tab, showing the organisms in which this sequence was identified.

12. **CLICK** on and **VIEW** the Descriptions **(j)** section, the Graphic Summary **(k)**, Alignment **(l)** and Taxonomy **(m)** sections.



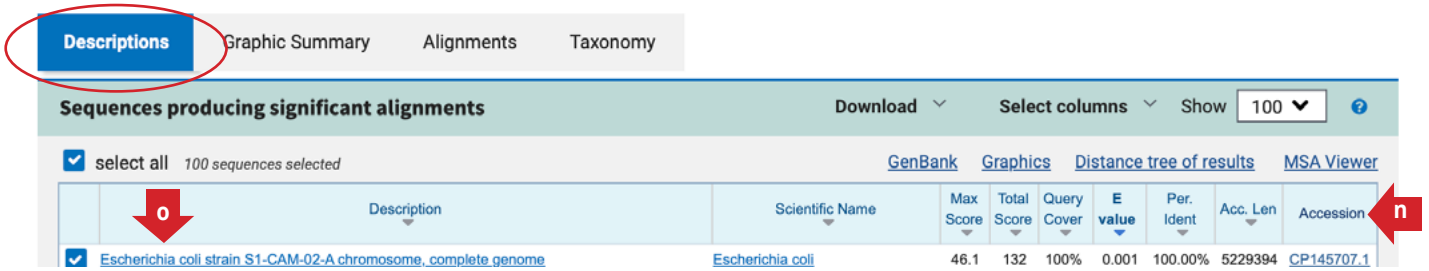
Appendix B: BLAST Bioinformatics Activity, continued

EXAMINE YOUR RESULTS

13. **REVIEW** the top BLAST result (first hit, first match). The top results should be a close match for your query (at least 20 bp), be in the organism *E. coli*, and be located in the *Lac*Z gene*. If this is not the case check that your query sequence and BLAST parameters are correct. In your lab notebook or BLAST document/spreadsheet **RECORD** the following: the accession number, the species, the type of sequence (complete genome, genome, complete sequence, partial sequence, etc.), the identities score, the range, and the gene.

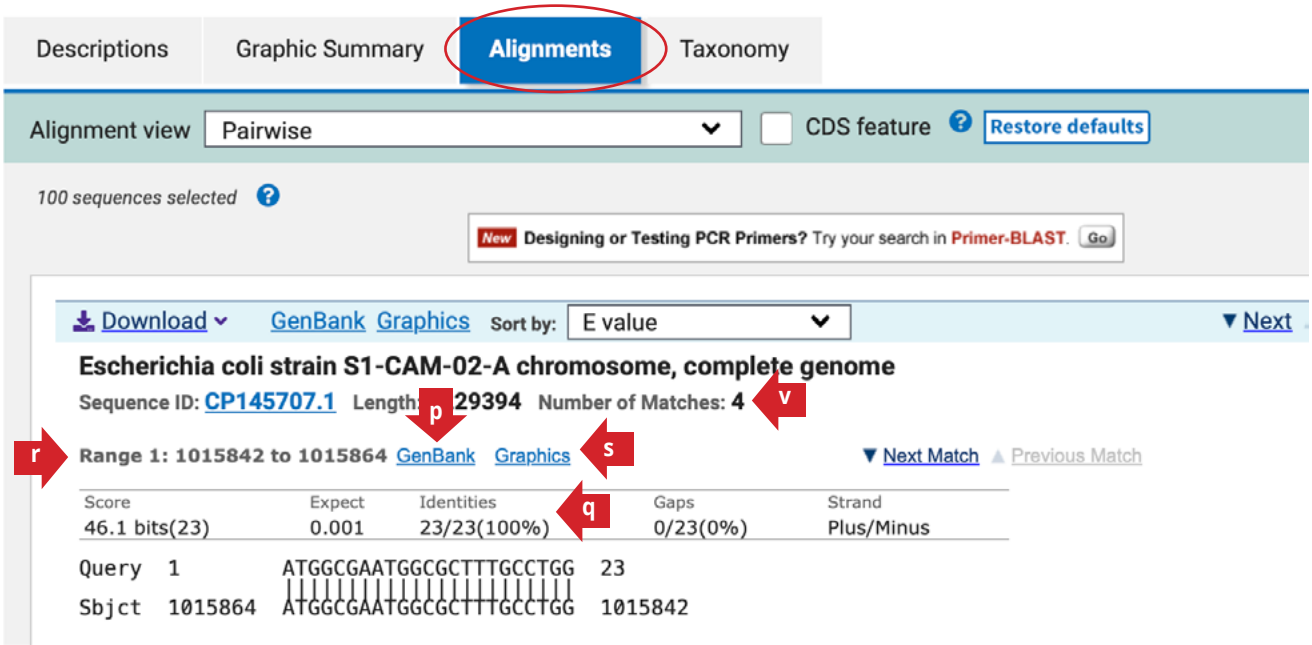
NOTE:
In some cases a GenBank sequence may not be annotated with gene names. If no gene name is available continue. In other cases the LacZ gene may also be referred to as Beta-Gal gene, the beta-galactosidase gene, or the β-galactosidase gene. In these cases, continue.

- The accession number **(n)** is displayed in the right most column in the description view.
- Species and sequence type **(o)** are usually given in the name which is displayed in leftmost column in the description view. You can also go to the alignment view and click on the GenBank link **(p)** for full information about the sequence.



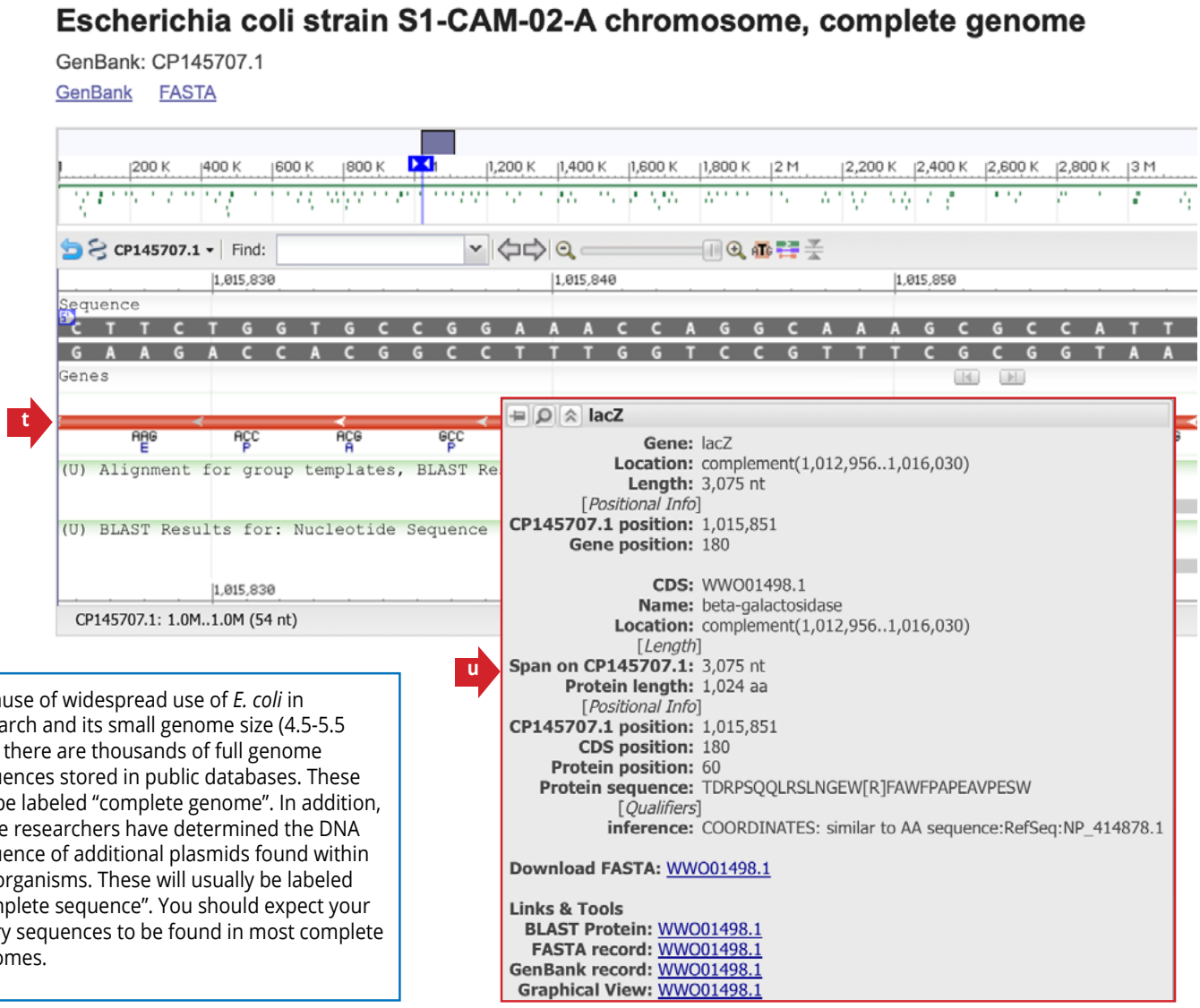
- The identities score reports the number of nucleotides involved in a match and the percentage of similarities within the match. To find these numbers go to the alignment view **(q)**.

- The range **(r)** is also present in the alignment view, just to the left of the genbank and graphic links.



Appendix B: BLAST Bioinformatics Activity, continued

- To find the gene, go to the alignment view and click on the graphics link **(s)**. This view provides additional biological information including genes. Hover your cursor over the red line **(t)** until the gene information box is displayed **(u)**. If a red line is not present, the region could be non-coding or the sequence could be not annotated.



Because of widespread use of *E. coli* in research and its small genome size (4.5-5.5 kbp) there are thousands of full genome sequences stored in public databases. These will be labeled "complete genome". In addition, some researchers have determined the DNA sequence of additional plasmids found within the organisms. These will usually be labeled "complete sequence". You should expect your query sequences to be found in most complete genomes.

14. **CHECK** for off target cut sites. **RETURN** to your original BLAST results page and the alignment tab. **CHECK** your first 100 sequences for hits *with more than 1 match*. The number of matches is displayed in the alignment view at the end of the second line **(v)**. **RECORD** notes and any instances where there are 2 or more matches with identities >23 and >85% in your lab book or BLAST document/spreadsheet. **USE** the same datapoints (accession number, species, identity score, gene etc) that you used in step 13 to describe these matches. ***Hint: To do this quickly click on the "Select All" box then switch to the alignment tab.***

15. **REPEAT** steps 3-14 for the three remaining query sequences. Place a double star next to any recorded hits (steps 13 and 14) that have identities that are 23/23 (100%). These are gene regions that would likely be modified by a CRISPR-Cas system containing your designed gRNA.

16. If time permits, **REPEAT** this exercise for a second gRNA or compare your results with your group or classmates to select a gRNA with the lowest risk of off target sites.

Appendix B: BLAST Bioinformatics Activity, continued

ANSWER THE FOLLOWING QUESTIONS:

1. (a) Did any of your query sequences exhibit an exact matches with 100% identity over a length of 23 base pairs? How many?

   (b) If a single gRNA had two (or more) query sequences with exact matches should it be considered for rejection due to potential off-target cut sites? Why or why not? If such instances occurred during your BLAST search, provide specific examples.

2. (a) Did any of your query sequence BLAST hits result in multiple matches? Among these matches, would any be considered high-risk for causing off-target cuts (with criteria such as >23 base pairs in length and >95% identity)?

   (b) Why might there be multiple matches from a single hit? (Hint: Considering genomic features that might contribute to sequence similarity within an *E. coli* genome.)

3. When assessing the risk associated with a potential off-target site, scientists consider several factors including the length of the match between the off-target and target sequences, the presence of gaps or mismatches, the pattern of gaps/mismatches, the presence of the PAM sequence, and the genomic location of the off-target site. Which of these factors did you examine in this exercise?