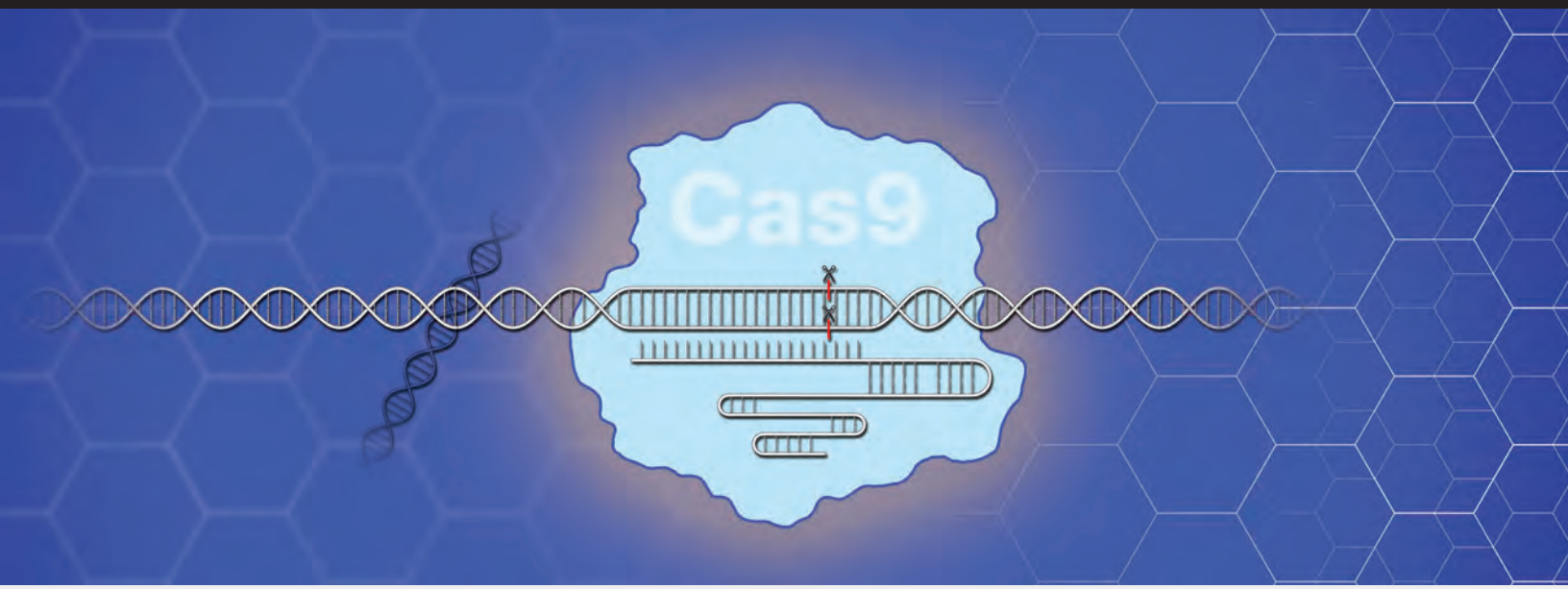
BIO-RAD Explorer



Out of the Blue CRISPR Kit

Catalog #12012608EDU

Instructor Guide

This product is not for self-administration.

For technical support, call your local Bio-Rad office, or in the U.S. call **1-800-4BIORAD** (1-800-424-6723) option 2.

BIO-RAD

Dear Instructor

The pace of discovery in life science and the power of human ingenuity are changing the lives and the futures of your students each and every day. Scientists can now harness a cell's innate ability to repair its DNA to modify a genome. Several approaches to genome editing have been developed, and among them, CRISPR (clustered regularly interspaced short palindromic repeats)-Cas9 stands apart for its accuracy, ease of use, and low cost. What was discovered as a form of prokaryotic immunity is now revolutionizing scientific research, agriculture, and medicine.

The Out of the Blue CRISPR Kit gives your students the tools to do true gene editing in the classroom. A first-of-its-kind learning activity, this kit will have your students exclaiming, "Wow! I did CRISPR!" Using familiar — and safe — reagents, techniques, and organisms, students will use CRISPR-Cas9 gene editing to disrupt the *lacZ* gene in *E. coli*. The resulting phenotype is readily visualized through blue-white screening, and students can use PCR to confirm the chromosomal edit using the optional Out of the Blue Genotyping Extension. As your students gain a deeper understanding of the unique capabilities and limitations of CRISPR-Cas9 technology, they are able to engage in more meaningful conversations around the promise, risks, and ethics of gene editing.

The activities included in this kit were developed in partnership with Sherry Annee, former president of the National Association of Biology Teachers and biotechnology teacher at Brebeuf Preparatory Academy in Indianapolis, IN, and Thomas Tubon Jr., Professor and Director of the Stem Cell Program at Madison Area Technical College in Wisconsin.

We strive to continually improve our curriculum and products, and your input is extremely important to us. We welcome your stories, comments, and suggestions.

Share your students' success on social media @bioradeducation.

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Kit Storage

When you receive the Out of the Blue CRISPR Gene Editing Kit:

1. Record storage location and batch numbers from the product labels.

2. Store the **Out of the Blue CRISPR Kit Reagent baggie** in the freezer (–20°C).

3. Store the **LB nutrient agar powder** and **LB broth capsules** at room temperature (~20°C).

4. Visit **bio-rad.com/outoftheblue** to download the most up to date instructor and student guides.

Technical Support is available at support@bio-rad.com or 1-800-4BIORAD, option 2.

Safety Guidelines

The Out of the Blue CRISPR Kit and refill pack contain kanamycin and spectinomycin, which may cause allergic reactions or irritation. Both antibiotics are added to LB agar media, which students will handle as part of the activity. Those with an allergy to these or similar antibiotics should consult with their physician before handling kit materials and reagents. Following the laboratory activities, place all bacteria plates and any materials that contacted bacteria in a 10% bleach solution for at least 20 min to decontaminate. Follow local regulations for further disposal recommendations.

The bacteria strain *E. coli* HB101-pBRKan contained in this kit is nonpathogenic and has been genetically modified to prevent its growth unless grown on an enriched medium. However, standard microbiological practices should be used.

Like restriction enzymes, Cas9 cuts DNA, but unlike restriction enzymes, Cas9 is programmable and can be directed to cut a particular sequence of DNA. The Out of the Blue CRISPR Kit laboratory activity involves using Cas9 to cut a particular bacterial gene, *lacZ*, which is present only in bacteria. Students will cut the *lacZ* gene and replace part of the sequence with a piece of DNA that will disrupt the gene. Disrupting *lacZ* in bacteria is commonplace in classroom and research laboratories alike. The approach in this laboratory activity builds upon these tried-and-true safe science practices and uses Cas9 as a new pair of molecular scissors.

Basic lab safety guidelines should be followed, including wearing gloves and safety goggles.

Kit Components

Each kit contains materials for 8 student workstations.

Item	Quantity
E. coli HB101-pBRKan, lyophilized	1 vial
Donor template DNA plasmid (pLZDonor)	215 µl
Donor template DNA and guide RNA plasmid (pLZDonorGuide)	215 µl
KIX Mix	263 mg
Spectinomycin powder	18 mg
L (+) arabinose, lyophilized	600 mg
LB nutrient agar powder	25 g
LB broth capsule, makes 50 ml LB broth	1 capsule
Inoculation loop, sterile, 10 µl	80
Petri dish, 60 mm, sterile	60
Microcentrifuge tube, natural, 2.0 ml	90
Transformation solution (50 mM $CaCl_2$, pH 6.1), sterile	15 ml
Easy Start Guide	1
Instructor Answer Guide	1

Required Materials (not included in this kit)	Quantity
100–1,000 µl adjustable-volume micropipet and tips	1–8
20–200 µl adjustable-volume micropipet and tips	4–8
2–20 µl adjustable-volume micropipet and tips	4–8
Balance with a range of 1 to 10 g	1
Autoclave or microwave oven	1
Thermometer (0–60°C)	1
Temperature-controlled dry bath or water bath	1
Foam floats, if using a water bath	8
Erlenmeyer flask or autoclavable bottle with cap, 500 ml	1*
Erlenmeyer flask or autoclavable bottle with cap, 1 L	1*
Autoclavable bottle with cap, 150–250 ml	1
Graduated cylinder, 500 ml	1
Deionized or distilled water	750 ml
Crushed ice and container (e.g., ice bucket or styrofoam cup)	8
Scissors	8
Permanent marking pens	8
Aluminum foil	
Household bleach, 10% solution	
Laboratory tape	

Recommended Materials (not included in this kit)	Quantity
Incubation oven or shaking incubator with dish shelf	1
Pipet controller and sterile serological pipets	1
Vortexer	1
Tube rack	8

* If your microwave cannot accommodate a 1 L flask, see Appendix C for modified LB agar plate preparation instructions. You will need two additional 500 ml flasks.

Out of the Blue CRISPR Kit

Ordering Information

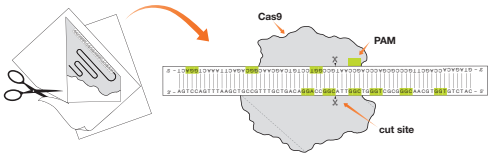
Catalog #	Description
Kits and Refill Packs	
12012608EDU	**Out of the Blue CRISPR Kit**
12012620EDU	**Out of the Blue CRISPR Kit Refill Pack**
12012607EDU	**Out of the Blue Genotyping Extension**
12012708EDU	**Out of the Blue Genotyping Extension Refill Pack**
17006070EDU	**Out of the Blue Genotyping Extension with Small Fast Blast Electrophoresis Pack**
17006284EDU	**Out of the Blue Genotyping Extension with Small UView Electrophoresis Pack**
17006081EDU	**Out of the Blue CRISPR and Genotyping Extension kits**
17006285EDU	**Out of the Blue CRISPR and Genotyping Extension kits plus Small UView DNA Electrophoresis Pack**
17006286EDU	**Out of the Blue CRISPR and Genotyping Extension kits plus Small Fast Blast DNA Electrophoresis Pack**
Consumables	
1660472EDU	**LB Nutrient Agar**, 500 g
1660412EDU	**LB Broth Capsule**, makes 50 ml LB broth
1660421EDU	**LB Nutrient Broth**, 10 ml
1660470EDU	**Petri Dishes**, 500
2239430EDU	**2 ml EZ Micro Test Tubes**, 500
1660471EDU	**Inoculation Loops**, 80
Equipment and Laboratory Supplies	
1660501EDU	**Mini Incubation Oven**, 120 V
17002944EDU	**Benchtop Shaking Incubator Starter Set**, 120 V
17002946EDU	**Benchtop Shaking Incubator Starter Set**, 230 V for Europe and the UK
12005504EDU	**Petri Dish Shelf for Shaking Incubator**
12011919EDU	**Mini Centrifuge**, 100–240 V
1660610EDU	**BR-2000 Vortexer**, 120 V
1660611EDU	**BR-2000 Vortexer**, 220 V for the EU
1660621EDU	**BR-2000 Vortexer**, 220 V for the UK
1660490EDU	**Professional Pipet Controller**
1660481EDU	**Green Racks**, set of 5 racks

Kit Activity Overview

Activity 1

Introduction to CRISPR-Cas9 Gene Editing Technology

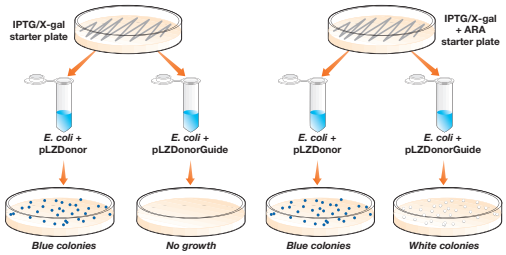
After a short reading, students use a paper model to walk through the steps of CRISPR-Cas9 DNA cleavage and homology directed repair. Then they use a mathematical model to demonstrate the far greater precision of CRISPR-Cas9 technology than that of restriction enzymes.



Activity 2

lacZ CRISPR Gene Editing Laboratory

Students edit the chromosomal *lacZ* gene in *E. coli* by transforming them with plasmid-based expression systems for donor template DNA and guide RNA. Then they use blue-white screening to calculate gene editing efficiency, and finally they write scientific explanations for their observations.



Capstone Activity

Identification and Bioinformatics Analysis of Cas9 Target Sites

Students apply their knowledge to identify candidate Cas9 target sites within genes linked to disease states. Then, using BLAST results as evidence, students rank their proposed target sites based on the risk for off-target editing.

Activity Timelines

The activities in this kit are designed to take three 90-minute or longer class periods as shown in Table 1. An alternative timeline for ~50 minute periods is outlined in Table 2.

Table 1. Suggested timeline for 90-minute or longer class periods meeting once or twice a week.

	Class Period 1	Class Period 2	Class Period 3
In-class work	Introductory lecture or other direct instruction Start Activity 1 Introduction to CRISPR-Cas9 Gene Editing Technology, Parts 1 through 4	Activity 2 *lacZ* CRISPR Gene Editing Laboratory, Part 2. Conduct Gene Editing	Start Capstone Activity
Outside-of-class work	Finish Activity 1 Activity 2 *lacZ* CRISPR Gene Editing Laboratory, Part 1. Answer Pre-Laboratory Questions	Students retrieve their plates after incubation and analyze the results Activity 2, Part 3. Answer Post-Laboratory Questions	Finish Capstone Activity

Table 2. Alternative timeline for daily ~50 minute class periods.

	Class Period 1	Class Period 2	Class Period 3	Class Period 4	Class Period 5
In-class work	Introductory lecture or other direct instruction Start Activity 1 Introduction to CRISPR/Cas9 Gene Editing Technology	Start Activity 2 *lacZ* CRISPR Gene Editing Laboratory, Part 2. Conduct Gene Editing	Continue Activity 2, Part 2	Finish Activity 2, Part 2. Start Activity 2, Part 3. Answer Post-Laboratory Questions	Begin Capstone Activity
Outside-of-class work	Finish Activity 1 Start Activity 2, Part 1. Answer Pre-Laboratory Questions			Finish Activity 2, Part 3. Answer Post-Laboratory Questions	Finish Capstone Activity

Curriculum Fit

Required prior knowledge

- Basic DNA, RNA, and protein structure and function
- Nucleotide base-pairing (how to write a complementary DNA or RNA strand)
- Central dogma (DNA → RNA → Protein → Trait)
- Simple probability calculations
- Key steps of bacterial transformation, including plasmid structure and function, antibiotic resistance selection markers, and heat shock
- How to culture bacteria on agar media
- How to use a micropipet

Concepts, topics, and skills

- **DNA, RNA, and protein structure and function** — Cas9 is a protein and a nuclease that cuts DNA as directed by guide RNA. The structures of these three components of the CRISPR-Cas9 system — protein, DNA, and RNA — are integral to its function
- **Central dogma** — students will disrupt the *lacZ* gene (DNA), which codes (via mRNA) for β-galactosidase (protein), an enzyme that allows *E. coli* to hydrolyze lactose, a milk sugar (trait). Students will edit the *lacZ* gene and observe a visible change in phenotype
- **Bacterial transformation** — this laboratory activity involves transforming bacteria with plasmids that code for CRISPR-Cas9 components. The process of bacterial transformation exploits the chemical properties of bacterial cell walls and DNA to introduce genetic information into an organism
- **Genetic engineering** — manipulating or engineering genomes to elicit a particular phenotype is the underlying goal of genetic engineering. Bacterial transformation and CRISPR gene editing are two techniques within this larger technology area
- **Bioinformatics** — students will practice using the Basic Local Alignment Search Tool (BLAST) to determine the relative risks of off-target gene editing for potential Cas9 target sites and evaluate the safety of using those target sites in CRISPR-based gene therapy
- **Ethical, legal, and social issues** — many broader issues are associated with the application of CRISPR technology because of the relative ease with which heritable genetic changes can be made

Preparation Instructions

Preparation step	Time required	When to begin preparation
Prepare LB agar plates	1–2 hr plus two days to dry plates	3–14 days before the activity
Rehydrate *E. coli*	5 min plus 8–24 hr incubation	2 days before the activity
Streak starter plates	20 min plus 24 hr incubation	24 hours before the activity
Dispense solutions	1 hr	Up to 3 days before the activity

Tips

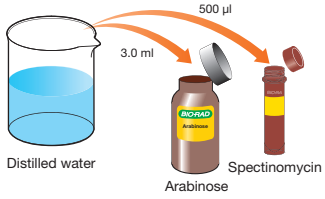
- Rehydrated *E. coli* must be incubated at 37°C for optimal results
- If you incubate starter plates at room temperature (20–25°C), incubate 72 hr. If you prepare starter plates over a weekend, incubate them at room temperature for 72 hr

Prepare LB Agar Plates 3–7 days before Activity 2

If your microwave cannot accommodate a 1 L flask, see Appendix C for modified plate pouring instructions.

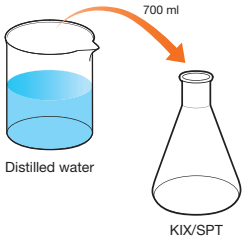
1. Label a 500 ml flask **KIX** and a 1 L flask **KIX/SPT**.

2. Add 3.0 ml distilled or deionized water to the vial of arabinose. Vortex or mix by pipetting for about 30 sec to begin dissolving. Briefly vortex or mix every few minutes until fully dissolved. Arabinose may take more than 10 min to fully dissolve.

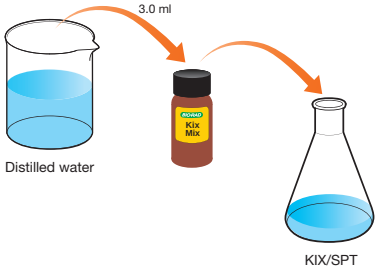


3. Add 500 µl distilled or deionized water to the vial of spectinomycin. Vortex or mix by pipetting until dissolved.

4. Add 700 ml distilled or deionized water to the flask labeled **KIX/SPT**.



5. Add 3.0 ml distilled or deionized water to the KIX Mix vial, recap, and shake to mix for 5 sec. Pour the KIX Mix slurry into the **KIX/SPT** flask.

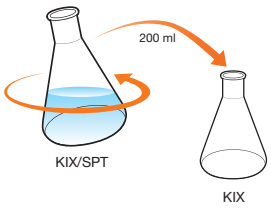


   Safety! The KIX Mix contains kanamycin, IPTG, and X-gal which are hazardous if inhaled. Do not remove the dry powder KIX Mix from the bottle without first adding water.

   Note: The KIX Mix will not fully dissolve and may appear clumpy.

6. Repeat step 5 at least twice to thoroughly rinse the KIX Mix vial.

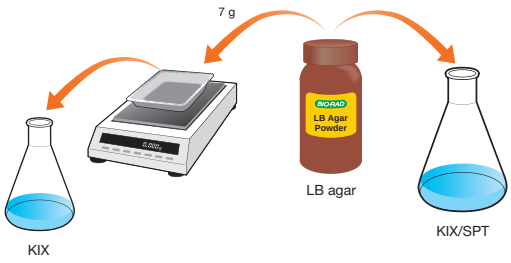
7. Swirl the solution for 20 sec or until the insoluble white powder is evenly suspended. Immediately pour 200 ml into the 500 ml **KIX** flask.



8. Add 7 g LB agar powder to the **KIX** flask. Add the remaining LB agar powder to the **KIX/SPT** flask.

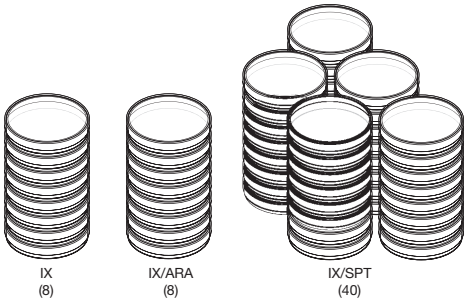
9. Autoclave both flasks for 30 min on a liquid cycle, or microwave each to boiling three times, taking care not to boil over.

   Note: If using an autoclave, check step 16 below before completing step 9.

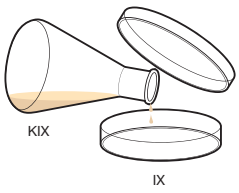


10. Label eight plates **IX** and eight plates **IX/ARA**. Label 40 plates **IX/SPT**.

    Important! KIX Mix contains the antibiotic kanamycin (K), which is used to select for *E. coli* HB101-pBRKan. All LB agar plates in this activity contain kanamycin. For simplicity, "K" and kanamycin are omitted from text and labels in all student instructional materials. If you would prefer to discuss the role of kanamycin with your students, replace **IX** labels with **KIX**.

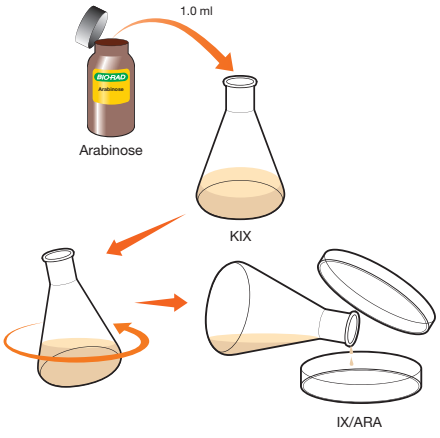


11. Quickly fill eight **IX** plates one-third to one-half full (~10 ml) with molten **KIX** LB agar.



12. Add 1.0 ml rehydrated arabinose to the remaining molten **KIX** LB agar. Swirl to mix, and fill eight **IX/ARA** plates about one-third to one-half full (~10 ml) with the molten agar.

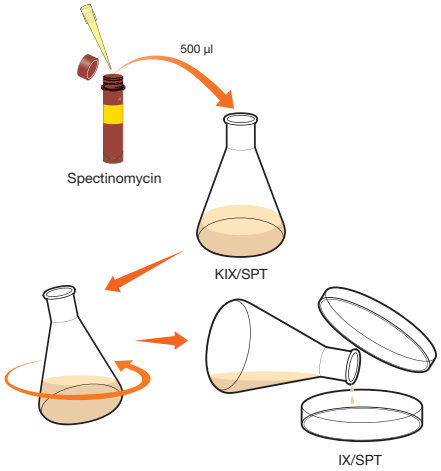
    Note: Rinse the KIX flask out right away to prevent solidified agar from sticking to the bottom.



13. Once the **KIX/SPT** flask can just be held comfortably (about 50°C), add 500 µl rehydrated spectinomycin. Swirl to mix, and fill at least 40 **IX/SPT** plates about one-third to one-half full (~10 ml) with the molten agar. Any additional poured plates can be used as replacements if needed.

    Note: Excessive heat (>60°C) will destroy spectinomycin. Do not add the spectinomycin until the agar has cooled enough to handle. However, the agar will solidify at 27°C so be sure to pour your plates before it has cooled too much. Placing your flasks in a 50°C water bath will help prevent the agar from cooling too quickly.

    Note: Rinse the KIX/SPT flask out right away to prevent solidified agar from sticking to the bottom.



14. Once solidified (~30 min), allow the plates to dry for two days at room temperature unwrapped and in the dark.

    Note: The **KIX** Mix additives are light sensitive, but the plates should not be wrapped while drying. Allowing plates to dry for two days improves the uptake of the liquid transformation samples in the student lesson.

15. After drying, wrap stacks of plates in aluminum foil to protect them from light and then wrap in plastic or put back in the original plastic sleeve. Store plates upside down and refrigerated at 4°C. Plates can be stored this way for up to two weeks before use.

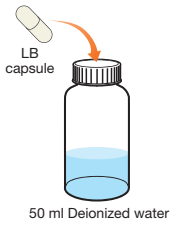
Rehydrate the bacteria 2 days before Activity 2

If you will be incubating starter plates at 37°C, rehydrate bacteria 2 days before Activity 2.

If you will be incubating starter plates at room temperature, rehydrate bacteria ***4*** days before Activity 2.

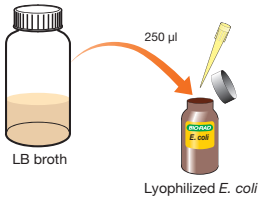
16. Add the LB broth capsule and 50 ml distilled or deionized water to a 150–250 ml bottle. ***Cap loosely*** and autoclave or microwave to boiling three times. Allow the broth to cool to room temperature. Tighten the cap and store refrigerated at 4°C. Warm to room temperature before use.

    Note: If autoclaving, this step can be combined with step 9 above.



17. Using a ***sterile*** pipet tip, add 250 µl room temperature LB broth to the vial of lyophilized *E. coli* HB101-pBRKan. Recap the *E. coli* vial and shake gently to resuspend the bacteria. Incubate the vial 8–24 hours at 37°C.

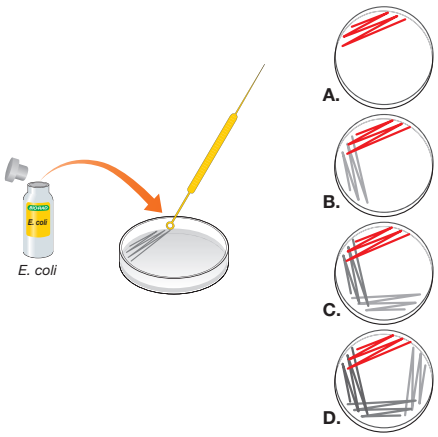
18. As a precaution against contamination, reboil the remaining LB broth once in the microwave with the cap loosened; tighten the cap and store at 4°C.



Streak and incubate starter plates 24 hours before Activity 2

19. Using a sterile plastic inoculation loop, streak the rehydrated *E. coli* HB101-pBRKan onto eight IX and eight IX/ARA plates.

    Important! Proper plate streaking technique to generate single colonies is essential for success. Follow the streaking pattern shown in steps A–D. First streak *E. coli* from side to side at the edge of one quadrant (A). Rotate the plate about a quarter turn. Pass the loop from side to side through the previous streaks multiple times, extending into the next quadrant (B). Repeat twice more (C, D).



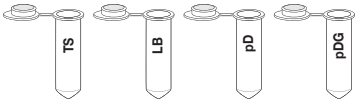
20. Incubate starter plates upside down in a 37°C incubator oven for 24 hours. Be sure the plates are kept dark in the incubator, but do not wrap them tightly in foil. Covering the incubator door in foil is effective.

    Note: Alternatively, starter plates may be incubated upside down at room temperature (20–25°C) in the dark for 72 hours.

Dispense solutions before Activity 2 and store at 4°C

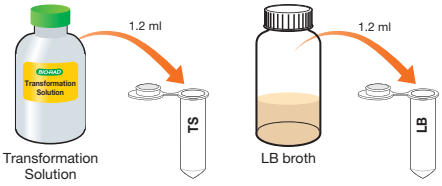
To prevent contamination, ***LB broth should be dispensed no more than 3 days before Activity 2.*** The other solutions can be dispensed up to 7 days before Activity 2.

21. For each of eight workstations, label a set of four 2 ml tubes the following: **TS**, **LB**, **pD**, and **pDG** (32 tubes total).



22. Add 1.2 ml transformation solution to each of the eight **TS** tubes.

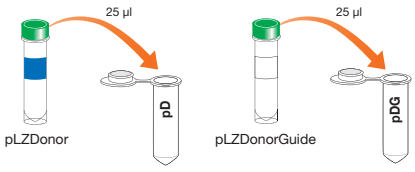
23. Add 1.2 ml sterile LB broth to each of the eight **LB** tubes.



24. Pulse-spin pLZDonor and pLZDonorGuide to collect the liquid at the bottom of the tubes.

25. Add 25 µl pLZDonor plasmid to each of the eight **pD** tubes.

26. Add 25 µl pLZDonorGuide plasmid to each **pDG** tube.



27. Store all solutions at 4°C until ready for use. Allow all reagents except transformation solution (**TS**) to come to room temperature before student use.

Prepare student workstations the day of Activity 2.

Student workstation

Materials	Quantity
Fresh *E. coli* IPTG/X-gal (**IX**) LB starter plate	1
Fresh *E. coli* IPTG/X-gal/ARA (**IX/ARA**) LB starter plate	1
IPTG/X-gal/spectinomycin (**IX/SPT**) LB plates	4
LB nutrient broth (**LB**)	1.2 ml
Transformation solution (**TS**) on ice	1.2 ml
pLZDonor plasmid (**pD**), 80 ng/µl	25 µl
pLZDonorGuide plasmid (**pDG**), 80 ng/µl	25 µl
100–1,000 µl adjustable-volume micropipet and tips (recommended)	1
20–200 µl adjustable-volume micropipet and tips	1
2–20 µl adjustable-volume micropipet and tips	1
Micro test tube, 2.0 ml	4
Yellow inoculating loop	8
Ice water bath with crushed ice	1
Permanent marking pen	1
Foam float (if using water bath)	1
Tube rack (recommended)	1
Waste cup	1

Common workstation

Materials	Quantity
Water bath or dry bath (holes filled with water) at 60°C	1
Incubator oven or shaking incubator with dish shelf at 37°C (recommended)	1
Lab tape	

Instructor Background

The Out of the Blue CRISPR-Cas9 System

The *lacZ* gene and blue-white screening

In this activity, students will use CRISPR-Cas9 technology to disrupt the *lacZ* gene, which occurs naturally in the chromosome of *E. coli* HB101-pBRKan, the strain of bacteria used in this kit. A gene in the bacterial *lac* operon, *lacZ,* encodes the enzyme β-galactosidase (β-gal), which allows *E. coli* to hydrolyze lactose. β-gal can also hydrolyze a colorless lactose analog, X-gal, leading to the formation of a blue pigment. The presence of blue or white colonies is a visual indication of successful *lacZ* gene editing: bacteria with functional *lacZ* turn blue when grown on media with X-gal; those with a disrupted, nonfunctional *lacZ* gene remain white (Figure 1).

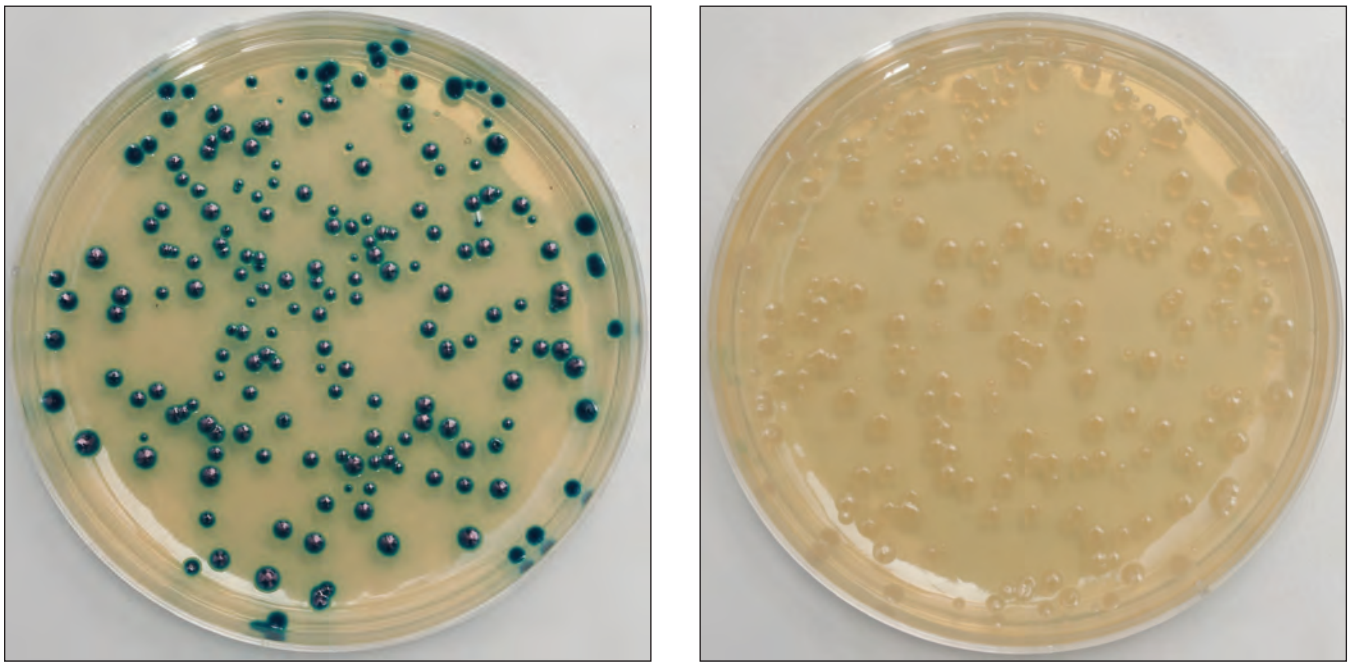


Fig. 1. *E. coli* plated on media containing X-gal. Bacteria with a functional *lacZ* gene are blue (left). Bacteria with a disrupted, nonfunctional *lacZ* gene are white (right).

CRISPR-Cas-mediated DNA breaks

The hallmark of CRISPR is the use of CRISPR-associated proteins, or Cas proteins, to target specific DNA sequences. Cas9 is the "classic" bacterial endonuclease used in CRISPR technologies, although many others have since been identified and engineered. Cas9 can be programmed to make double-strand breaks in DNA at a specific target site. The target site is determined by a short RNA molecule, called a guide RNA, that forms a complex with Cas9.

DNA repair by homology directed repair

Once a chromosome cut is made by Cas9, it must be repaired in some way or the bacteria will die. The bacteria used for this activity do not natively have the ability to repair breaks in DNA, but they have been engineered to express the enzymes for a DNA repair process called homology directed repair (HDR). The genes encoding the HDR pathway are under the control of an arabinose-inducible promoter. In order to repair a DNA break, HDR requires donor template DNA with homology to the sequences flanking the cut site. Donor template DNA is supplied on the plasmids included in the kit (see below).

E. coli HB101-pBRKan

The bacteria included in this kit, HB101-pBRKan, have been engineered to express the Cas9 enzyme. They carry a plasmid containing genes for homology directed repair (HDR) under the control of an arabinose-inducible promoter. The plasmid carries a kanamycin resistance gene as a selection marker.

The sgRNA and donor template DNA

Students will transform *E. coli* HB101-pBRKan with one of two plasmids, each of which contains a spectinomycin resistance gene:

- pLZDonor — (control) includes a donor template DNA sequence that will be used by the HDR machinery to fix double-strand DNA breaks. The donor sequence includes a stop codon that will impair *lacZ* function when inserted
- pLZDonorGuide — includes both the donor template DNA sequence from pLZDonor and a sequence that codes for the single guide RNA (sgRNA). Once transcribed, the sgRNA will guide the Cas9 protein where to cut *lacZ*

The donor template sequence contains a stop codon that will terminate the translation of the *lacZ* gene. Bacteria that have undergone gene editing using this donor template DNA will not express functional β-gal but will continue to grow into white colonies. Bacteria with a double-strand DNA break in their chromosome but without an active mechanism to repair the break will die.

How the components work together

In these activities, different media additives are used to bring about the various experimental outcomes (Table 3).

- All plates contain the antibiotic kanamycin as a selection marker for *E. coli* HB101-pBRKan
- All plates contain IPTG (isopropyl β-D-1-thiogalactopyranoside) and X-gal (5-bromo-4-chloroindol-3-yl-β-D-galactopyranoside) for blue-white screening. IPTG is a nonmetabolizable analog of galactose that induces the expression of the *lac* operon, including *lacZ* which codes for β-gal
- Arabinose induces expression of the HDR enzymes. Students may be confused about why the experimental plates (IX/SPT) do not contain arabinose. Because the entire gene editing process, including HDR, is completed before students plate their bacterial samples, it is not necessary for the experimental plates to contain arabinose
- Plates containing spectinomycin select for bacteria transformed with pLZDonor or pLZDonorGuide

Table 3. Media additives for starter and experimental LB agar plates.

Plate Name	**Kanamycin*** Selection marker for the bacterial strain	**IPTG** Induces expression of β-gal	**X-gal** Hydrolyzed by β-gal to produce a blue pigment	**Arabinose** Induces expression of HDR system	**Spectinomycin** Selection marker for pLZDonor and pLZDonorGuide
IX	✔	✔	✔		
IX/ARA	✔	✔	✔	✔	
IX/SPT	✔	✔	✔		✔

*** Important!** All plates used in this activity contain the antibiotic kanamycin (K), which is used to select for the bacterial strain. For simplicity of the activity, "K" and kanamycin are omitted from text and labels in all student instructional materials. If you would prefer to discuss the role of kanamycin with your students, replace IX labels with KIX and explain its purpose to your students.

Students will begin the activity with bacteria grown on two different starter plates (**IX** and **IX/Ara**).

- All bacteria express Cas9
- Bacteria grown on **IX** plates do not express the enzymes required for HDR (repair system OFF)
- Bacteria grown on **IX/ARA** plates do express the enzymes required for HDR (repair system ON)

Bacteria from both starter plates will be transformed with either pLZDonor or pLZDonorGuide (see Figure 2).

- Bacteria transformed with pLZDonor will not have the sgRNA needed to guide Cas9 to cut *lacZ*. Therefore, no gene editing will occur, and transformants will be blue regardless of whether the repair system is ON or OFF
- Bacteria transformed with pLZDonorGuide will have both the sgRNA and donor template DNA
  - If the HDR system is not expressed (arabinose is absent), Cas9-mediated cutting will occur but no DNA repair will occur, and the cells will die. No growth will be observed
  - If the HDR system is expressed (arabinose is present), Cas9-mediated cutting will take place and the HDR machinery will use the donor template DNA from pLZDonorGuide to patch the cut and introduce a stop codon into the *lacZ* gene. Transformants will be white
- Immediately following transformation, Cas9-mediated cutting and repair processes will begin. These processes will mostly be complete prior to spreading on **IX/SPT** plates
- Spectinomycin in the **IX/SPT** agar media selects for those bacteria that were successfully transformed

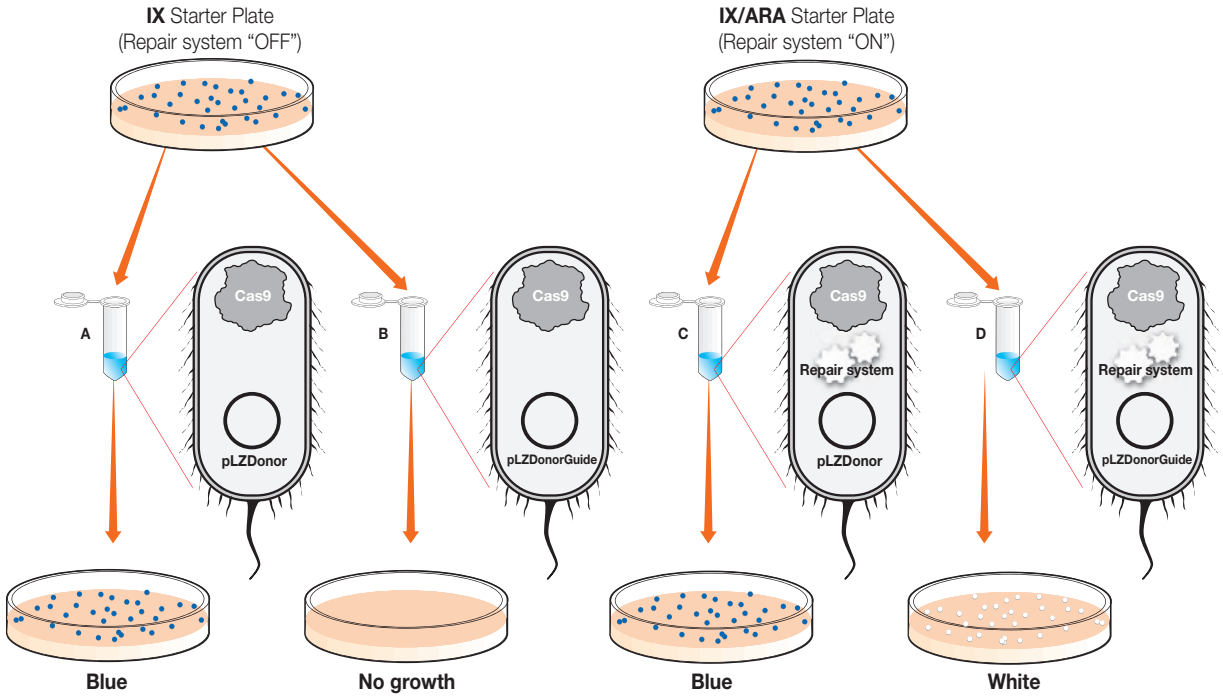


Fig. 2. Transformations performed with this activity. Expected results are shown.

Activity 1

Introduction to CRISPR-Cas9 Gene Editing Technology

The activities in this kit focus on the process of CRISPR-Cas9 gene editing technology and provide students with a limited explanation of the bacterial adaptive immune system from which it was derived. Provide additional background information from this guide, a video, a news article, or direct instruction to introduce these concepts as appropriate for your students. Additional resources are listed in the appendices.

Goals: Students understand the steps of Cas9-mediated DNA cleavage and can predict the location of cleavage when given an sgRNA sequence. Students can use mathematical models to explain the relative specificity of restriction enzymes and Cas9 to cut DNA.

	Teaching tips and notes
Part 1. Simulate the Molecular Mechanism of Cas9 DNA Cleavage	• This activity is designed to be mostly student driven • Students should use sgRNA 1 and DNA Strip 1 • The DNA sequence in the activity is a portion of the *E. coli* chromosomal *lacZ* gene • Formative assessment opportunity: check students' predicted cut sites for accuracy and address any misconceptions you discover • Option: provide a larger DNA sequence from a notable gene and an sgRNA sequence of your choice. Have students predict the cut site
Part 2. Design the Guiding Region of an sgRNA	• Students should use sgRNA 2 and DNA Strip 2 • Formative assessment opportunity: check students' sgRNA sequences for accuracy and address any misconceptions you discover. See the Instructor Answer Guide included in the kit for the correct sgRNA sequence • Option: provide a larger DNA sequence from a notable gene and instruct students to write an sgRNA sequence that would cut within the provided DNA sequence
Part 3. Compare the Specificity of DNA Cutting Tools	• The questions in this part ask students to assume that the human genome is a random sequence of A, T, C, and G. In actuality, many nucleotide sequences are repeated in the human genome • Discuss the potential impact of sequence repeats, such as paralogs, orthologs, and *Alu* repeats, on the specificity of CRISPR-Cas9 technology • Formative assessment opportunity: check student calculations and interpretations. See the Instructor Answer Guide included in the kit
Part 4. Design Donor Template DNA for DNA Repair	• In discussions with students, emphasize the difference between gene repair, fixing the sequence of a gene for function, and DNA repair, fixing molecular breaks in DNA • Formative assessment opportunity: have students display their donor template DNA strands before taping them to the cut DNA strips. Ask other students to review • Option: have students find the full *lacZ* gene sequence using the NCBI database and propose an sgRNA target sequence and donor template DNA sequence to insert an in-frame stop codon

Activity 2

lacZ CRISPR Gene Editing Laboratory

Goal: Students conduct chromosomal gene editing and understand the expected outcomes of a CRISPR-Cas9 experiment.

	Teaching tips and notes
Part 1. Answer Pre-laboratory Questions	• All plates in this activity contain kanamycin as a selection marker for the strain of bacteria used in this kit. For simplicity, "K" and kanamycin are omitted from text and labels in all student instructional materials. If you would prefer to discuss the role of kanamycin with your students, replace IX labels with KIX labels during the preparation • Gene editing in this activity is chromosomal and is mostly complete before students plate and incubate their samples. Plating and incubating allows colony formation and a visible phenotype • Students may be confused about why arabinose is not required in the experimental plates. Arabinose is required for DNA repair which begins immediately after transformation. Once the cut in the bacterial chromosome has been repaired, the repair machinery is no longer needed. All daughter cells generated during colony formation will have the chromosomal edit which will not be cut again by Cas9. • Formative assessment opportunity: before students proceed to Part 2, review their answers in Student Guide Tables 3 and 4 to check for misconceptions
Part 2. Conduct Gene Editing Protocol	• Prior to the heat shock step, it is crucial that students keep their samples on ice at all times unless actively transferring solutions • If you will be continuing on with the Out of the Blue Genotyping Extension, students will need to keep their bacterial plates. Visit **bio-rad.com/outoftheblue** or see the Out of the Blue Genotyping Extension Instructor Guide for details • If your students will not be analyzing their plates immediately after incubation, store them refrigerated (4°C) for up to two weeks until analysis
Part 3. Answer Post-laboratory Questions	• No colonies should grow on plate B because the bacteria have active Cas9 with guide RNA but lack expression of the engineered DNA repair system because they were grown without arabinose. In rare cases students may observe a few white colonies growing on plate B. These bacteria have likely undergone a rare deletion event in which some portion of the *lacZ* gene has been deleted and the chromosomal DNA has been left intact • The CRISPR-Cas9 gene editing system in this kit is highly efficient, but it is not 100% efficient. Some students will find blue colonies on plate D which arise from bacteria with *lacZ* genes that were not modified • Option: if continuing with the genotyping extension, have students analyze the genotype of a colony that has experienced a deletion event • Formative assessment opportunity: ask students to explain the roles of IPTG, X-gal, and spectinomycin in the experiment • Formative assessment opportunity: have students make predictions about expected bacterial colony color if they subcultured a sample on plates that contained IPTG and X-gal but no spectinomycin

Capstone Activity

Identification and Bioinformatics Analysis of Cas9 Target Sites

Goal: Students discover medical applications of CRISPR and explore real factors that affect the risk of off-target effects in disease therapies. Students reinforce BLAST skills and review basic design requirements for CRISPR experiments.

Introduction

The potential for off-target effects is a major factor in ethical discussions involving CRISPR-mediated gene editing technology. The choice of target cut site and required guide RNA sequence is critical to reducing the risk of off-target effects. Students who are first learning about CRISPR technology may not immediately recognize these limitations nor appreciate the necessity for risk assessment during gene therapy design and development. In this activity your students will use BLAST to evaluate candidate Cas9 target cut sites in a therapeutically relevant gene based on the risks of off-target effects.

The results of this activity are qualitative and meant to guide students to appropriate questions, not answers, about the real risks associated with CRISPR applications. This is an activity in which there are no correct answers, only well justified arguments. It provides a space for students to explore, try, fail, and iterate toward a defined goal.

Notes on the activity design and limitations

The reality of assessing guide RNA sequences for off-target effects is complex. Many free online tools are available to help scientists find and rank candidate guide RNA sequences, but their algorithms and scoring criteria are complex and/or proprietary. In this activity, students use BLAST not only to practice using such an important tool, but also because the reality of off-target sequences is made obvious. Thus, while this activity is not the workflow that scientists typically use, it does help students to consider some basic design elements.

- BLAST has limitations when dealing with short sequences and prioritizes sequences with sequential nucleotide alignment over sequences with intermittent mismatches or alignment gaps. As a result, BLAST may ignore some sequences with high potential to be off-targets
- BLAST cannot correctly utilize a wildcard nucleotide, N, in short nucleotide searches. Instead the algorithm ignores all nucleotides downstream of N, which in this case includes the PAM sequence
- A comprehensive off-target search should include the protospacer sequence with each of the four PAM combinations (5'-AGG, 5'-TGG, 5'-CGG, and 5'-GGG). Because BLAST is unable to correctly handle N in a single query sequence, four separate BLAST searches, one for each PAM sequence combination, are necessary to truly evaluate the off-target potential of a candidate target sequence. To fit within the timeframe of a classroom activity, this activity has been simplified and students use only one sequence
- The Human Reference Gene Database, which students are using in this activity, includes only gene sequences. Therefore, all query results will be from gene sequences, which helps to underscore for students the potential biological impact of an off-target cut. Off-targets outside of genes will not be found, but the results will be much easier for students to interpret
- As a further extension, have students develop a list of design requirements for a software developer to develop an sgRNA design tool

	Teaching tips and notes
Part 1. Identify and Catalog Target Sequences	• Assign student groups and provide each with one disease state information sheet (Coronary Artery Disease, Sickle Cell Disease, or Cystic Fibrosis) • The provided gene sequences are limited in length to reduce the number of candidate target sites • All target site candidates should be 23 nucleotides long (protospacer sequence plus a PAM at the 3' end) and written as a single-strand sequence 5' to 3' • Remind students that the guiding sequence of sgRNA is complementary to a target sequence. By selecting a target site, the sgRNA sequence is also specified. Therefore, “designing” the guiding region of an sgRNA is essentially the same as selecting a specific target site.
Part 2. Perform BLAST Search for Off-Target Sequences	• BLAST query sequences must be written 5' to 3' but can be from either DNA strand • Students are using BLAST to find off-target DNA sequences that match or partially match their candidate DNA sequence. They are not searching for RNA sequences • Option: if there are multiple students in a group, have each member query their candidate sequences with one of the four PAM sequence options (5'-AGG, 5'-TGG, 5'-CGG, and 5'-GGG). See notes on the activity design and limitations above
Part 3. Evaluate Candidate Target Sequences	• Students may encounter accession numbers with large sequences that contain several independent genes, each of which has its own accession number. In effect, these genes are doubled in the database. For simplicity, it may be best for students to ignore these large sequences when analyzing their results Example: NG_000007.3 is a long DNA fragment containing five globin genes including *HBB*, which is separately contained in NG_059281.1 • When developing criteria, students might consider: – whether a potential off target sequence is adjacent to a PAM sequence in the correct orientation – how many potential off target sequences completely match the target sequence – how many potential off target sequences match at least 80% of the target sequence (or some other percentage) – the location and pattern of mismatches between a potential off target and target sequences (far or close to the PAM) • Students criteria should address two aspects of the risk for off targets: the level of risk that a particular off target site might be cut, and the quantity of risky off targets there are for an individual target site.

Appendix A

CRISPR Gene Editing Structured Student Debate

The potential for CRISPR gene editing technology to address problems in medical, agricultural, and environmental settings is matched only by its potential for misuse. Though the ethical debates surrounding genetic engineering are not new, the fact that CRISPR can be more accurate and easier to perform than other technologies has reignited concerns about its applications. A student debate is a great way to get your students to engage with the ongoing public discussions about CRISPR technology and to reinforce their understanding of the scientific facts.

Key areas of debate

- At what point does the risk of unintended off-target editing outweigh the potential for a cure or treatment of a debilitating disease?
- Should germline CRISPR gene therapy be allowed? Such changes could eliminate genetic disease, but might there be unintended consequences to a population?
- If CRISPR can be used to edit out disease, should it also be used for genetic enhancement, to change eye color, intelligence, athletic ability, or height?
- Who should decide which genetic changes are allowed or prohibited?

Day One — Set the Stage

1. Find a recent article that describes a particular use case of a CRISPR-based gene editing technology. Use an article that explains the specific techniques used.

2. Randomly divide the class into two groups and assign one group to support the use of CRISPR gene editing technology in the article and the other to oppose the use.

3. Explain the format of the debate and have each team pick a captain.

Days Two–Five — Student Research

4. Provide the article to your students. Have them conduct additional research on the case and record their findings in the Pro/Con Data Sheet on page 22; optionally assign for homework.

5. Have teams compile research from all members.

6. Allow teams to write 4-min opening statements and assign spokespersons.

Day Six – The Debate

Table 4. Debate format.

Section	Time	Requirement
Opening statement	4 min	Proponents present an opening statement supporting the resolution
Break	2 min	Opponents assemble a list of questions they believe show holes in proponents' arguments
Questions	2 min	Opponents present questions
Opening statement	4 min	Opponents present an opening statement rebutting the subject
Break	2 min	Proponents assemble a list of questions they believe show holes in opponents' arguments
Questions	2 min	Proponents present questions
Rebuttal	2 min	Proponents present answers to opponents' questions
Rebuttal	2 min	Opponents present answers to proponents' questions
Closing arguments	3 min	Opposing view
Closing arguments	3 min	Supporting view

Grading Rubric

	4	3	2	1
Opening statements	Eloquent, very well organized, researched, and presented	Well organized, researched, and presented	Somewhat organized, researched, and presented	Lacking organization, partially correct research, not well presented
Questions	Questions were thoughtful, raised legitimate concerns, were research based, and were well presented	Questions were somewhat thoughtful, raised some concerns, and were well presented	Questions were not research based, did not raise legitimate concerns, or were not well presented	Questions were unrelated to the subject, did not raise legitimate concerns, or were not well presented
Rebuttal	Students used research data and conclusions to directly refute the questions	Students used research data and conclusions to partially refute the questions	Students used research data and conclusions improperly when attempting to refute the questions	Students did not refute the questions
Closing statements	Closing statement was eloquent, very well organized, researched, and presented	Closing statement was well organized, researched, and presented	Closing statement was somewhat organized, researched, and presented	Closing statement lacked organization, used partially correct research, and was not well presented
Working as a team member (as ranked by other team members)	Fully participated and contributed to the team	Participated and contributed to the team	Partially participated, somewhat helpful to the team	Little participation, little help to the team

Pro/Con Data Sheet

Make a list of benefits of the use of CRISPR gene editing technology mentioned in the article (include references).

Make a list of negative consequences of the use of CRISPR gene editing technology mentioned in the article (include references).

Note research information that can refute the claims of the other debate group. Include these in your opening or closing statement.

Appendix B

CRISPR-Cas9 and a Microbial Immune System

Animals have complex immune systems that involve the coordinated activities of multiple cell types, organs, and signaling systems to recognize and respond to active infections. Only recently researchers have discovered that prokaryotes, bacteria and archaea, also have a form of adaptive immunity that allows them to recognize and respond to viral infections: the CRISPR-Cas system.

Some prokaryotic genomes contain short, palindromic DNA sequences that are repeated many times, with unique "spacer" sequences between the repeats. These repeated sequences are called "clustered regularly interspaced short palindromic repeats," or CRISPR (Figure 3). Each palindromic repeat is followed by short segments of spacer DNA that match DNA sequences found in bacteriophage genomes (bacteriophage are viruses known to infect bacteria). Groupings of CRISPR-associated (Cas) genes are also found next to CRISPR sequences, and these Cas genes encode enzymes that cut DNA in specific places, like precise molecular scissors.

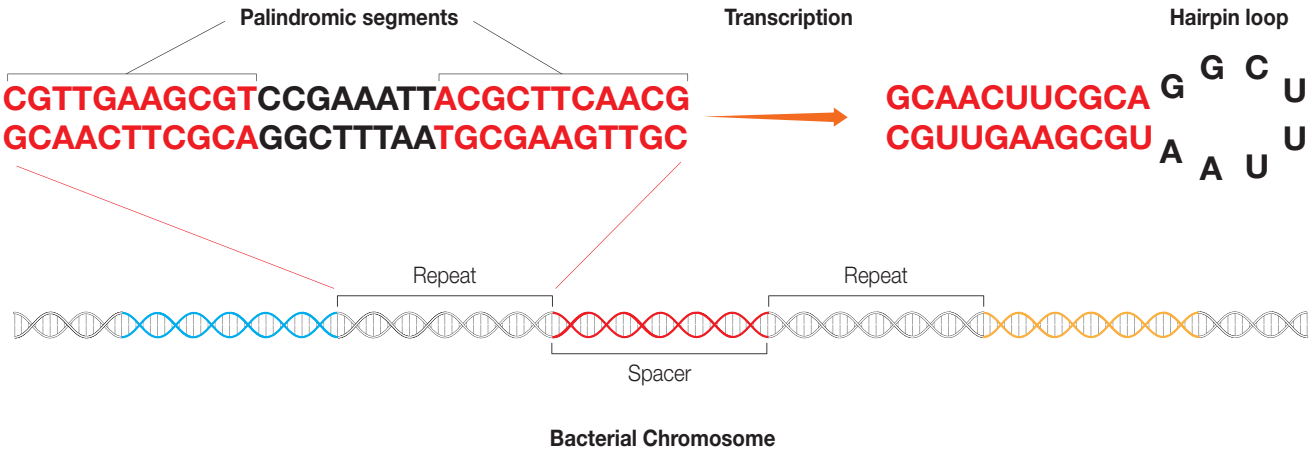


Fig. 3. A region of a bacterial genome with clustered regularly interspaced short palindromic repeats (CRISPR). Prokaryotic genomes contain clusters of palindromic repeats. These repeats are separated (regularly interspaced) by short segments of spacer DNA. These CRISPR segments occur alongside groupings of CRISPR-associated (Cas) genes, which encode the Cas enzymes responsible for cutting DNA. In a palindromic repeat, the sequence of nucleotides is the same in both directions, and upon transcription, the resulting RNA forms a multi-hairpin loop structure.

In 2007, researchers put these curious facts together to demonstrate that the CRISPR and Cas genes work together to provide a form of immune response to bacteriophage invasion (Barrangou et al. 2007), which involves three phases (Figure 4):

- **Cutting and capture** — when bacteria are infected by a virus, they use components of their CRISPR system to cut up the invading viral DNA and insert pieces of it (spacers) into their own genome as a "memory" of the infection
- **Monitoring** — bacteria transcribe the spacers into RNA that can form a complex with the Cas enzyme. These complexes monitor the cell for any DNA sequence complementary to the RNA
- **Defense** — if matching (viral) DNA is encountered, the spacer RNA-Cas complex binds to it and cuts the viral DNA to prevent it from replicating. This halts the viral infection

Using this system, bacteria can collect sequences from many different infecting viruses to create a “library.” Since the CRISPR sequence is contained in genomic DNA, it is passed on to each generation, and the library continues to develop over time.

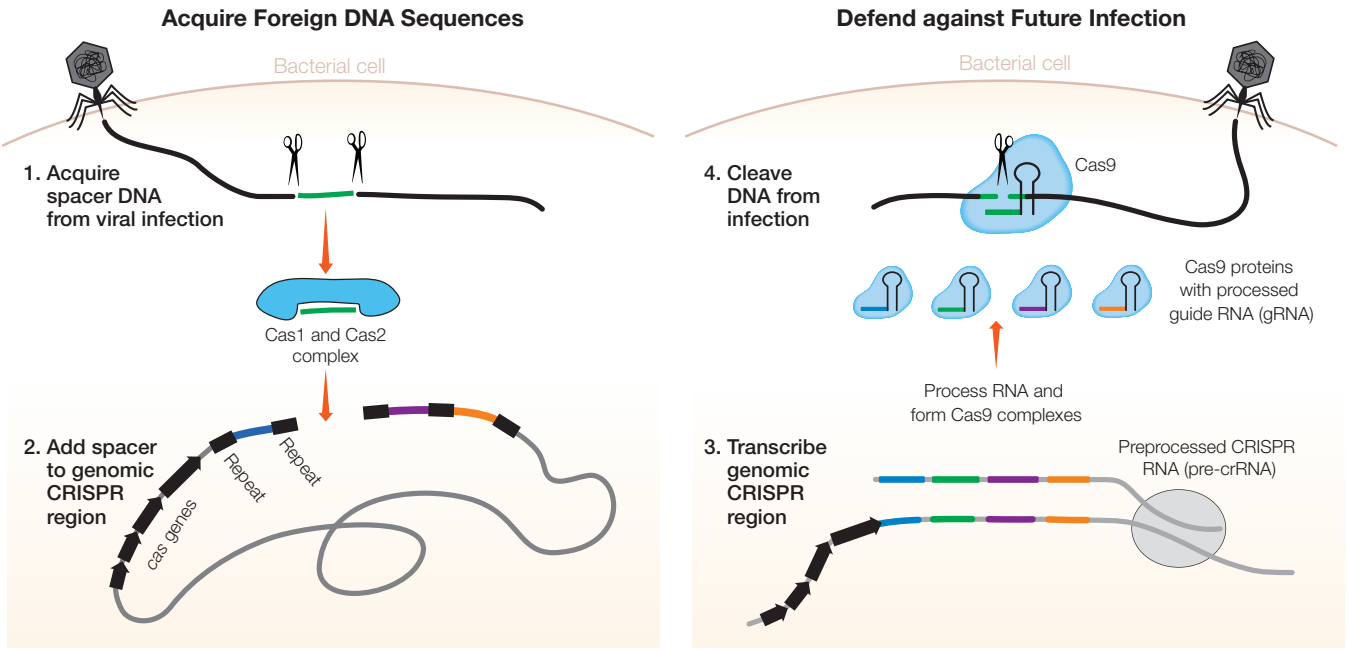


Fig. 4. The CRISPR-Cas9 microbial defense system. 1, the Cas1-Cas2 enzymes of the microbe recognize foreign DNA and cut out a segment. **2**, the Cas1-Cas2 enzymes insert the DNA segment into the CRISPR region of its own genome as a spacer. **3**, a spacer sequence is transcribed and then linked to a Cas9 protein. **4**, upon reinfection by the same invader, the CRISPR-Cas9 complex can recognize the foreign DNA sequence and cut it to prevent complete infection.

Appendix C

Small Microwave LB Agar Plate Preparation Instructions

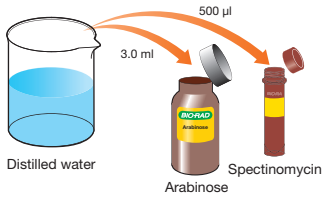
If you are using a microwave that cannot accommodate a 1 L flask, use these modified instructions, which utilize 500 ml flasks in the microwave, to prepare LB agar plates. Then continue with normal preparation.

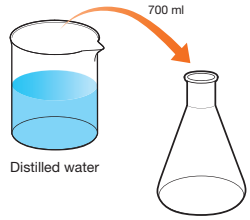
1. Label a 500 ml flask **KIX** and two 500 ml flasks **KIX/SPT**.

2. Add 3.0 ml distilled or deionized water to the vial of arabinose. Vortex or mix by pipetting for about 30 sec to begin dissolving. Briefly vortex or mix every few minutes until fully dissolved. Arabinose may take more than 10 min to fully dissolve.

3. Add 500 µl distilled water to the vial of spectinomycin. Vortex or mix by pipetting until dissolved.

4. Add 700 ml distilled or deionized water to an empty 1 L flask.

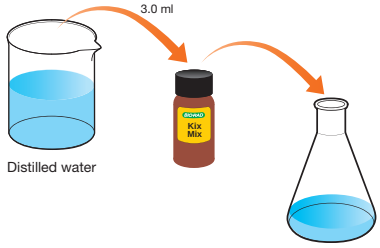




5. Add 3.0 ml distilled water to the KIX Mix vial, recap, and shake to mix for 5 sec. Pour the KIX Mix slurry into the 1 L flask.

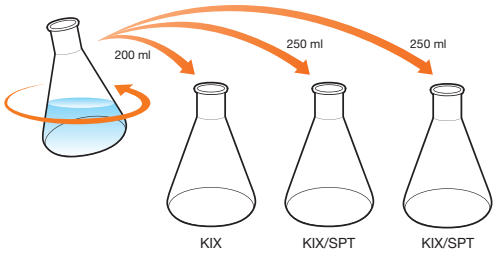
   Safety! The KIX Mix contains kanamycin, IPTG, and X-gal which are hazardous if inhaled. Do not remove the dry powder KIX Mix from the bottle without first adding water.

   Note: The KIX Mix will not fully dissolve and may appear clumpy.

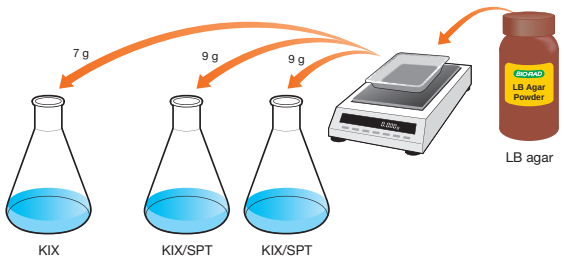


6. Repeat step 5 at least twice to thoroughly rinse the KIX Mix vial. Inspect the vial to make sure all the contents were transferred to the flask.

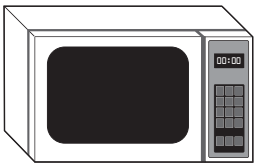
7. Swirl the solution for 20 sec or until the insoluble white powder is evenly suspended. Immediately pour 200 ml into the **KIX** flask. Swirl the suspension again quickly and immediately pour 250 ml into each **KIX/SPT** flask.



8. Add 7 g LB agar powder to the **KIX** flask. Add 9 g LB agar powder to each of the **KIX/SPT** flasks. Swirl to mix.

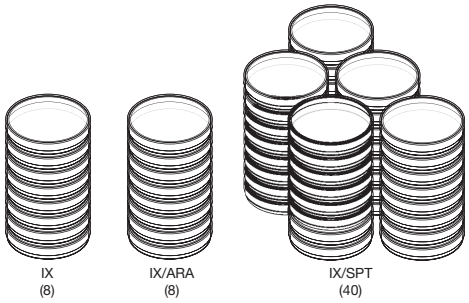


9. Microwave each flask to boiling three times, taking care not to boil over. Start with 3 min and add 1 min intervals as needed.

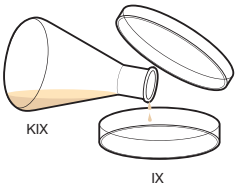


Note: Use a hot pad or mitt when handing the hot flask. Be careful not to allow the LB/agar to boil over. Use a lower power microwave setting and watch it carefully. Allow the molten agar to cool slightly before swirling to prevent boil over.

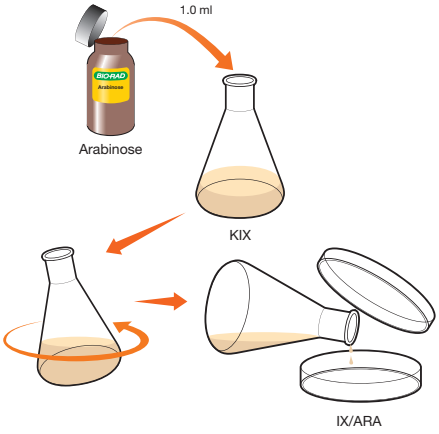
10. Label eight plates **IX,** eight plates **IX/ARA**, and 40 plates **IX/SPT**.



11. Quickly fill eight **IX** plates one-third to one-half full (~10 ml) with molten **KIX** LB agar.

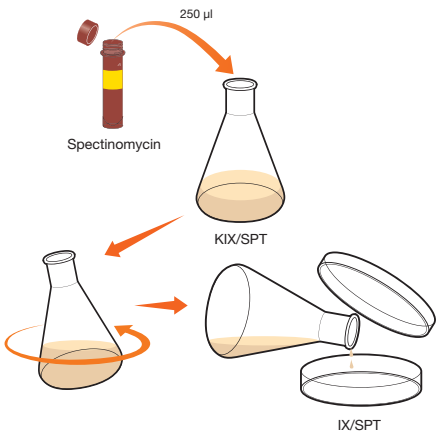


12. Add 1.0 ml rehydrated arabinose to the remaining molten **KIX** LB agar. Swirl to mix and fill eight **IX/ARA** plates about one-third to one-half full (~10 ml) with the molten agar.



13. Once the **KIX/SPT** flasks can just be held comfortably (about 50°C), add 250 µl rehydrated spectinomycin to each **KIX/SPT** flask. Swirl to mix and fill at least 40 **IX/SPT** plates one-third to one-half full (~10 ml) with molten **KIX/SPT** LB agar. Any additional poured plates can be used as replacements if needed.

    Note: Excessive heat (>60°C) will destroy spectinomycin. Do not add the spectinomycin until the agar has cooled enough to handle. However, the agar will solidify at 27°C so be sure to pour your plates before it has cooled too much. Placing your flasks in a 50°C water bath will help prevent the agar from cooling too quickly.



14. Once solidified (~30 min), allow the plates to dry for two days at room temperature unwrapped and in the dark.

    Note: The **KIX** Mix additives are light sensitive, but the plates should not be wrapped while drying. Allowing plates to dry for two days improves the uptake of the liquid transformation samples in the student lesson.

15. After drying, wrap stacks of plates in aluminum foil to protect them from light and then wrap in plastic or put back in the original plastic sleeve. Store plates upside down and refrigerated at 4°C. Plates can be stored this way for up to two weeks before use.

Resources

Visit **bio-rad.com/outoftheblue** for additional classroom resources.

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