# 2025 Inaugural CRISPR Course Workshop

7-11 JULY 2025 • Cambridge, MA

Hosted By CGER and TSU

# Welcome!

We are delighted to welcome you to the 2025 Inaugural CRISPR Course Workshop, a collaborative effort between the Center for Genome Editing and Recording (CGER) and Tennessee State University (TSU). We are thrilled to have you join our community of educators dedicated to diversifying the STEM workforce.

Our vision is to create a vibrant environment in which scholars across educational and socioeconomic levels—from underrepresented students to university and high school educators—engage without barriers.

As many of you know, the scientific enterprise as a whole severely lacks researchers of color which extends to the genome editing field. The lack of representation is particularly salient in a field with profound ethical ramifications—diverse voices should be heard at all stages of scientific work, from initial research to real-world translation. To ensure that the field continues to devote attention to conditions impacting minority groups, and for that attention to be thoughtful, it will be important for scientists of color to be present in the rooms where decisions are being made. By increasing representation, we can foster innovation and ensure that research addresses the needs and concerns of all communities.

Our workshop is inspired by the acclaimed CRISPR course developed by the Innovative Genomics Institute, co-founded by Jonathan Weissman and Jennifer Doudna. Since 2017, this course has been offered to UC Berkeley students, and we are excited to bring its spirit and content to you. Over the coming days, you'll hear from leading experts, participate in hands-on lab experiments, and explore the journey of CRISPR—from its origins as a bacterial immune system to its groundbreaking applications in genome editing and beyond.

Our hope is that you will leave this workshop empowered and inspired—ready to start new CRISPR-based courses or integrate this cutting-edge science into your own classrooms and institutions. Together, we can spark curiosity and excitement in the next generation of scientists, especially among students from non-dominant groups, and help shape a more inclusive future for genome editing.

Thank you for joing us here. We look forward to learning, collaborating, and making discoveries with you!

With best regards,

Jonathan Weissman  Maxine Wang  Masami Hasa

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# Agenda

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| **Sunday, July 6, 2025** | |
| | Arrival and hotel check-in |
| **Monday, July 7, 2025** | |
| 6:30 - 9:00 am | Breakfast at hotel |
| 9:00 - 9:30 am | Registration at Whitehead Institute |
| 9:30 - 10:45 am | Course Introduction |
| | Maxine Wang, Whitehead Institute, & Sonali Roy, Tennessee State University |
| 10:45 - 11:00 am | Coffee break |
| 11:00 - 12:00 pm | CRISPR Intro and CRISPR Applications |
| | Laralynne Przybyla, Arena BioWorks |
| 12:00 - 1:30 pm | Lunch and Networking Session |
| 1:30 - 5:00 pm | Lab Section - CRISPR Immunity |
| 6:00 pm | Welcome Dinner |
| **Tuesday, July 8, 2025** | |
| 6:30 - 9:00 am | Breakfast at hotel |
| 9:00 - 9:30 am | Arrival to Whitehead Institute |
| 9:30 - 10:45 am | Lab Section - Bioinformatics (designing a guide RNA) |
| | Sonali Roy, Tennessee State University |
| 10:45 - 11:00 am | Coffee break |
| 11:00 - 12:00 pm | Science Communication |
| | Mary Williams, American Society of Plant Biologists |
| 12:00 - 1:30 pm | Lunch and Networking Session |
| 1:30 - 5:00 pm | Lab Section - Cas9 *In Vitro* Assay |
| **Wednesday, July 9, 2025** | |
| 6:30 - 9:00 am | Breakfast at hotel |
| 9:00 - 9:30 am | Arrival to Whitehead Institute |
| 9:30 - 10:45 am | Engineering stable antibody expression using CRISPR/Cas9-mediated gene integration |
| | Shelbe Johnson, Massachusetts Institute of Technology |
| 10:45 - 11:00 am | Coffee break |

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|---|---|
| 11:00 - 12:00 pm | Plant Epigenetics<br>Mary Gehring, Whitehead Institute |
| 12:00 - 1:30 pm | Lunch and Networking Session |
| 1:30 - 5:00 pm | Lab Section - Cas9 *In Vivo* Bacteria Kit |

**Thursday, July 10, 2025**

| | |
|---|---|
| 6:30 - 9:00 am | Breakfast at hotel |
| 9:00 - 9:30 am | Arrival to Whitehead Institute |
| 9:30 - 10:45 am | Launching Pilot CRISPR Course at TSU<br>Sonali Roy, Tennessee State University & Ali Taheri, Tennessee State University |
| 10:45 - 11:00 am | Coffee break |
| 11:00 - 12:00 pm | Synthetic microbe-to-plant communication channel to monitor the soil quality<br>Alice Boo, Massachusetts Institute of Technology |
| 12:00 - 1:30 pm | Lunch and Networking Session |
| 1:30 - 5:00 pm | Lab Section - CRISPR Application in Plants |

**Friday, July 11, 2025**

| | |
|---|---|
| 6:30 - 9:00 am | Breakfast at hotel |
| 9:00 - 9:30 am | Arrival to Whitehead Institute |
| 9:30 - 10:45 am | Curriculum Pitch Preparation Time |
| 10:45 - 11:00 am | Coffee break |
| 11:00 - 12:00 pm | Curriculum Pitch Part 1 |
| 12:00 - 1:30 pm | Lunch and Networking Session |
| 1:30 - 3:00 pm | Curriculum Pitch Part 2 |
| 3:00 - 3:15 pm | Closing Remarks<br>Maxine Wang, Whitehead Institute, & Sonali Roy, Tennessee State University |

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| **WiFi Access** | **MIT:** | **Whitehead Institute:** |
| | MIT GUEST | Wibr-guest |

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|---|---|
| **Emergency Number** | **MIT Police**<br>617-253-1212 |

## Location and Venues

**Hotel:**

The Kendall Hotel
350 Main St
Cambridge, MA 02142

**Seminars:**

Whitehead Institute
455 Main St
Cambridge, MA 02142

**Lab Section:**

MIT Building 68
31 Ames St
Cambridge, MA 02139

# Curriculum Pitch Overview

During the week of the workshop, you will team up with a colleague to develop a curriculum pitch for how you will either integrate CRISPR into an existing course or create a completely new class at your home institution. We hope that the connections you make during this workshop becomes a network where learnings can be shared and synergies identified to enable potential collaborations.

On Friday each team will have 30 minutes to present and strategize how you would like to integrate your learnings from this workshop. It will be important to identify potential challenges you and your colleagues will need to overcome. We envision 10-15 minutes of presentation, followed by discussions. There is no structure to the presentation, you may present on any aspects of a curriculum whether it be on:

- Learning outcomes
- Module roadmaps
- Active learning strategies
- Assessment schemes
- Implementation of lab or project components
- Highlighting ethical and regulatory considerations

Questions to think about:

- Alignment – Do activities, assessments, and outcomes line up?
- Pedagogical creativity – Evidence-based teaching methods that boost engagement for historically excluded students.
- Feasibility at your institute – Realistic budgeting, safety, and equipment plans; adaptability to differing lab resources.
- Community impact – Opportunities for students to connect gene editing to local agriculture, medicine, or policy.
- CRISPR knowledge depth – up-to-date technology developments, acknowledgment of limitations.

# Notes

# Bios

## Organizers

**Jonathan Weissman**
CGER Director; HHMI Investigator; Professor of Biology, MIT; Member of Whitehead Institute

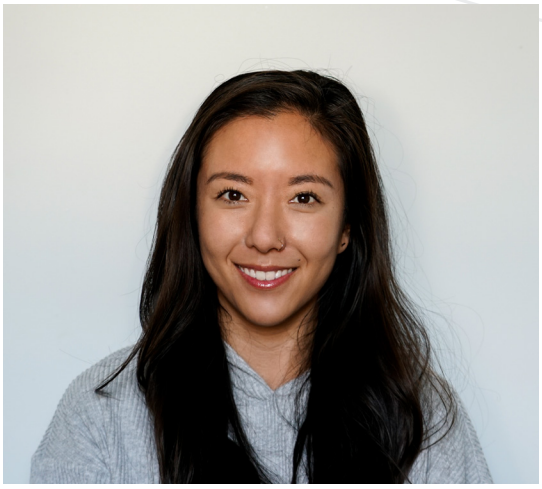
Jonathan Weissman earned his bachelor's degree in physics from Harvard College in 1988, graduating summa cum laude. He next took on a PhD in physics from MIT, where he studied under biochemist Peter S. Kim, and from 1993-1996 he completed a postdoctoral fellowship at Yale in the lab of Arthur Horwich. For the past 24 years he has held faculty positions in the Departments of Cell and Molecular Pharmacology and Biochemistry & Biophysics at the University of California, San Francisco, until joining Whitehead Institute in 2020. He also co-leads the Laboratory for Genomic Research, funded by GlaxoSmithKline, to drive development of CRISPR-based therapeutics.

**Sonali Roy**
Assistant Professor, TSU

Sonali Roy is an Assistant Professor in the College of Agriculture at Tennessee State University in Nashville. She earned her Ph.D. from the John Innes Centre, UK, where she studied auxin regulation in root nodule and arbuscular mycorrhizal symbiosis. Her postdoctoral research at the Noble Research Institute in Oklahoma led to the discovery of peptide hormone families involved in macronutrient signaling and nodule development.

Since 2018, Sonali has incorporated CRISPR-Cas9 technologies into her research program using *Medicago truncatula* to functionally dissect regulatory genes involved in legume root development and nitrogen-fixing symbioses. Her lab combines gene editing, transcriptomics, and peptide signaling assays to explore the molecular mechanisms underlying legume-microbe interactions. She currently leads two federally funded research projects focused on applying CRISPR-based approaches in legumes and holds a teaching grant supporting CRISPR pedagogy in undergraduate classrooms.

Sonali is also committed to scientific communication and serves as a Reviewing Editor at The Plant Cell (2017–2019).



**Maxine Wang**
CGER Program Manager

Maxine Wang received her BS in Molecular Environmental Biology from UC Berkeley and is now an HS Chau Scholar at Caltech. Maxine has developed a strong background in strategic organizational development, scientific outreach, and scientific program management. Her experience spans research and leadership roles within both academia and industry, where she has managed large-scale, multidisciplinary genomics collaboration and led educational initiatives to promote diversity in STEM. Maxine's expertise has enabled her to foster impactful collaborations between a wide-range of teams, ensuring that innovative technologies like CRISPR are accessible and relevant to a broad scientific community. She is dedicated to empowering underrepresented minorities and building inclusive scientific networks through hands-on education and strategic partnership-building.

**Masami Hazu**
CGER Curriculum
Development Lead

Masami Hazu earned her Ph.D. in Molecular Biology from the California Institute of Technology, where her research focused on the molecular mechanisms of membrane protein biogenesis. She holds a BSc from Nagoya University in Japan and an MSc from the University of Sheffield in the UK. Masami's international academic journey has shaped her commitment to fostering inclusive and accessible STEM education. At Caltech, she led multiple initiatives to support diversity and mentorship, including chairing her department's women's group for three years and co-founding both the DEI Representatives program and the Diversity in BBE group. She is passionate about scientific outreach and mentorship, and continues to advocate for equity in science through community engagement.

**Mandana Sassanfar**
Senior Lecturer; Director of
Outreach, MIT

Mandana Sassanfar holds a BS and MS in Biochemistry from the University Pierre and Marie Curie, Paris VI, and a PhD in Biochemistry from Cornell University. After completing her postdoctoral work at the Harvard School of Public Health and the Massachusetts General Hospital, she spent several years working in industry and teaching at Harvard College before joining MIT in 2002. Mandana spearheads a number of outreach activities primarily for low income students and those from institutions with limited research opportunities. She supervises multiple summer programs, organizes field trips to MIT, teaches a number of lab courses, and coordinates training opportunities for high school science teachers. In 2012, she became a Fellow of the American Association for the Advancement of Science and the Massachusetts Academy of Sciences, and earned the Dean's Education & Advising Award from the MIT School of Science. She was also the President of the National Association of Academies of Science between 2011 and 2013.

# Bios cont.

## Speakers

**Laralynne Przybyla**
Disease Mechanisms and Models Team Lead, Arena Bioworks

Laralynne Pryzbyla leads the Disease Mechanisms and Models Team at Arena BioWorks to integrate human genetics, disease-relevant models, and high-throughput screening assays with a goal of uncovering the mechanisms of human disease to identify opportunities for therapeutic intervention. Prior to this role, Dr. Przybyla was an Associate Professor at UCSF and the UC Scientific Director at the Laboratory for Genomics Research, a joint industry-academia hybrid institute between UCSF, UC Berkeley, and GSK. She obtained her PhD in Biology from MIT and has experience leading technology development teams across both academia and industry to accelerate development of novel therapeutics across disease areas including neurodegenerative disease, autoimmune disorders, oncology, cardiovascular disease, and kidney disease.

**Mary Williams**
Features Editor at American Society of Plant Biologists

Mary Williams studied Biochemistry at Berkeley (BA) and Plant Molecular Biology at Rockefeller (PhD). She did a postdoc at Berkeley with Ian Sussex, and then spent 14 years as a Biology Professor at Harvey Mudd College in Claremont, California. In 2009 she started working at the American Society of Plant Biologists as Features Editor of the journal Plant Cell and the developer of Teaching Tools in Plant Biology. Her passion lies in making it a little bit easier for students of all ages to understand plants and plant science research.

**Shelbe Johnson**
Chemical Engineering PhD candidate, MIT

Shelbe Johnson received her Bachelor's degree from Georgia Institute of Technology. She is currently a NSF graduate research fellow in Brandon DeKosky's Lab at the Ragon Institute of MGH, MIT, and Harvard. She is particularly passionate about developing and using new technologies to improve human health across the globe. One of her projects involves leveraging CRISPR-Cas9 to engineer stable antibody expression.

**Mary Gehring**
HHMI Investigator; David Baltimore Chair; Whitehead Institute; Professor of Biology and Biological Engineering, MIT

Mary Gehring began her scientific career at Williams College, earned her doctorate from University of California Berkeley in 2005, and continued her studies as a postdoctoral researcher with Steven Henikoff at the Fred Hutchinson Cancer Research Center. Gehring came to Whitehead Institute in 2010 and was named the Thomas D. and Virginia W. Cabot Career Development Professor by MIT in 2011. In 2020 she was named the Landon T. Clay Career Development Chair at Whitehead Institute. In 2023, Gehring was named the Inaugural David Baltimore Chair in Biomedical Research. In 2024, she was selected as an Investigator of the Howard Hughes Medical Institute.

**Alice Boo**
Postdoctoral Scholar, MIT

Alice Boo is a Postdoctoral Associate in Professor Chris Voigt's lab in the Department of Biological Engineering at MIT. She completed both her MEng in Biomedical Engineering in 2017 and her Ph.D. in Synthetic Biology in 2022 from Imperial College London. In the lab, she is engineering a synthetic microbe-to-plant communication channel to monitor the soil quality of agricultural crops. She is passionate about all forms of communication, especially visual communication and graphic design, and helping people communicate their research through visuals. Outside the lab, Alice can be found working on eclectic sets of projects from painting to woodworking or gardening.

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# Lab Introduction

Get ready for an exciting, immersive week exploring the fascinating world of CRISPR-associated (Cas) proteins and their groundbreaking applications. This intensive, week-long laboratory section is designed to give you hands-on experience with running CRISPR experiments, and enable you to teach these core concepts and the broader implications of genome editing technologies as a whole.

## Course Structure and Logistics

Our lab sessions will primarily take place in the afternoons, each lasting approximately 3–4 hours. While breaks are built into the schedule, please be aware that some days will be action-packed and fast-paced. To keep things running smoothly, certain steps will be pre-prepared for you; however, all protocols and procedures will be laid out in this lab manual for your reference.

Throughout the week, we will be using three commercially available kits, each accompanied by a comprehensive teacher guide. These guides provide in-depth information about kit components and experimental procedures. If you'd like to dive deeper, digital versions of all kit manuals are available on the workshop drive (both USB and cloud).

## What You'll Learn

Originally discovered as a form of adaptive immunity in bacteria, CRISPR systems have rapidly evolved into powerful tools for genome editing. This course will guide you through this remarkable transition from natural biology to transformative technology. Each day will center around a key theme:

Day 1: CRISPR-Cas in nature

Day 2: Programmable DNA cleavage with Cas9 in an *in vitro* assay

Day 3: *In vivo* genome editing with Cas9 in *E. coli*

Day 4: Applications of CRISPR in plant research

## Beyond the Bench

To support your learning, we've included worksheet exercises and additional teaching materials to help you navigate experimental design, data interpretation, and troubleshooting. We'll also engage in thoughtful discussions about the ethical and policy considerations surrounding CRISPR and its applications.

We're excited to have you on this journey with us—let's make the most of this incredible week of discovery, experimentation, and collaboration!

# Lab Structure

| Topic | Kits | Page |
|---|---|---|
| CRISPR Immunity | | 18 |
| Cas9 *in vitro* assay | miniPCR Chopped! | 30 |
| Genome editing with Cas9 in *E. coli* | LabAids: The Power of CRISPR<br>Biorad: Out-of-the-blue CRISPR kit | 38 |
| Designing and cloning guide RNA | | 62 |

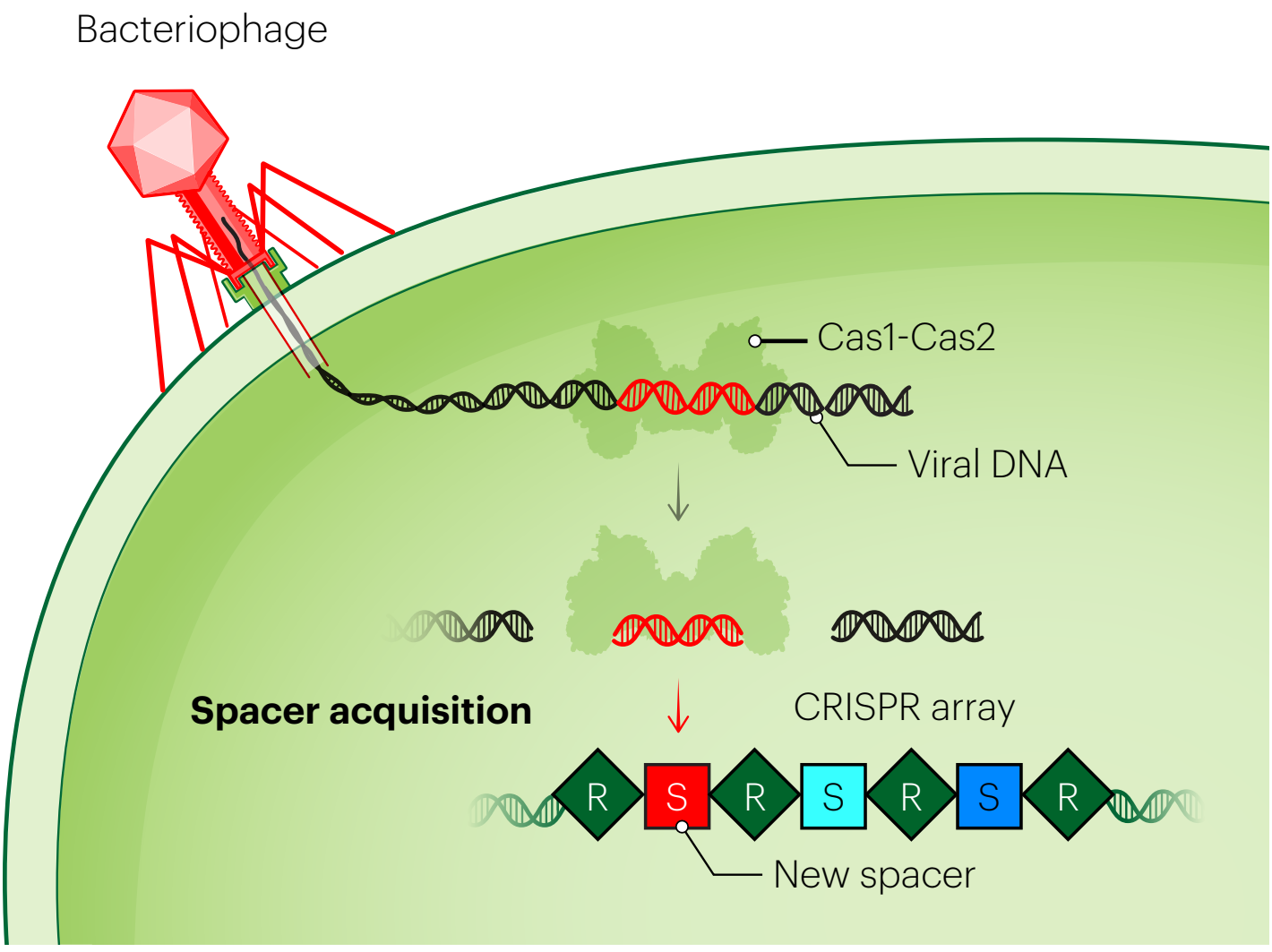
## Note to Instructors

This is a dual-purpose lab manual designed to support both students (you, during this workshop) and instructors. We've included dedicated instructor preparation sections—clearly marked in grey boxes (like here)—to help facilitators set up experiments in advance. While this manual provides all the essential information needed to run the lab, we encourage you to consult the vendors' instructor guides for the commercial kits. These guides offer in-depth details, as well as additional resources on ethics and real-world applications of CRISPR, which we will only briefly address during the workshop given time limitations.

# Part 1: CRISPR Immunity

Before scientists harnessed the power of CRISPR-Cas systems, they were elucidating their natural role in prokaryotic adaptive immunity. Unlike other immune defenses in prokaryotes, CRISPR provides a form of molecular memory by recording past viral infections. This stored information acts like a "mugshot," enabling the cell to recognize and target the virus if it invades again.

In this lab session, we will explore the mechanism behind CRISPR immunity. You will work with *E. coli* engineered with an inducible plasmid that expresses the Cas1 and Cas2 proteins—key players in the acquisition phase. These proteins form a complex that captures fragments of invading DNA, called spacers, and integrates them into the bacterium's CRISPR array (see figure below). By examining the bacterial DNA before and after inducing Cas1–Cas2 expression, we can observe spacer acquisition and identify the sequences of newly incorporated spacers.



## Friday 7/4

### Instructors preparation: Streak out plates of bacteria

Materials:

- Glycerol stock *E. coli* (DH5alpha) with pCas1+Cas2
- 10x LB + Streptomycin agar plates
- Sterile inoculation loops
- Incubator set at 37˚C

Protocol:

1. Take ten LB + Streptomycin agar plates and label them ***E. coli* DH5a pCas1+2**.
2. Using a sterile inoculation loop, touch the top of the glycerol stock to scrape some bacteria off.
3. Gently spread the bacteria all over the agar plate.
4. Repeat **steps 2 - 3** for the other nine plates.
5. Turn the plates lid-side down, and leave them to incubate at 37˚C, overnight.
6. In the morning, single colonies should be visible. A single colony should look like a white dot growing on the solid medium. If the bacterial growth is too dense and you do not see single colonies, re-streak onto a new agar plate to obtain single colonies.
7. The plates can be stored in a 4˚C refrigerator for up to one week.

# Part 1: CRISPR Immunity cont.

## Saturday 7/5

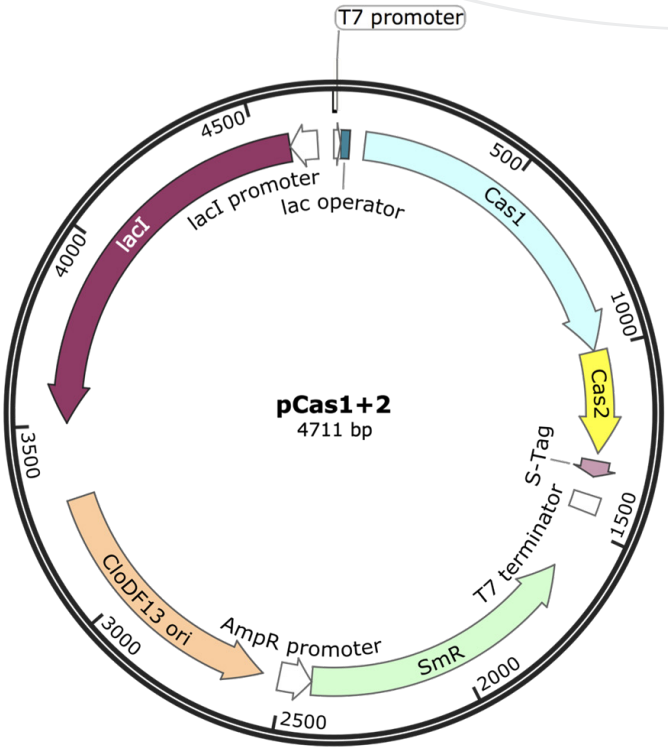
### Instructors preparation: Aliquoting materials for bacterial culture

Materials:

- Sterile LB media
- Sterile 1000x streptomycin solution
- Sterile 20% arabinose solution
- Sterile 0.1 M IPTG (Isopropyl β-d-1-thiogalactopyranoside) solution
- 10x 15-mL conical tubes
- 30x 1.5-mL microcentrifuge tubes
- Pipettes
- Pipette tips

Protocol:

1. For each of the 10 student groups, prepare one 15-mL conical tube and label it **LB**.
2. For each of the 10 student groups, prepare three 1.5-mL microcentrifuge tubes and label them **Strep**, **Ara**, and **IPTG** (30 tubes total).
3. Aliquot 12 mL sterile LB media to the tubes labeled **LB**.
4. Aliquot 15 µL 1000x streptomycin solution to the tubes labeled **Strep**.
5. Aliquot 110 µL 20% arabinose solution to the tubes labeled **Ara**.
6. Aliquot 10 µL 0.1 M IPTG to the tubes labeled **IPTG**.
7. Store the aliquoted **Strep**, **Ara**, and **IPTG** tubes at -20˚C until the class. Store the **LB** at room temperature.




*Cas1 and Cas2 plasmid map (Addgene: 72676)*

Today we will inoculate cultures with *E. coli* containing the Cas1-Cas2 expression plasmid. We will take initial samples so that we know what the CRISPR locus looks like to begin with. We will culture and induce the expression of Cas1 and Cas2 with IPTG and arabinose. The cells will begin to acquire spacers. The uninduced culture will serve as a control, showing that without Cas1 and Cas2, spacers are not acquired.

## Saturday 7/5 (prepared in advance)

### Student task: Start liquid culture of *E. coli* and induce the expression of Cas1 and Cas2 on the plasmid

Materials (for each pair):

- Agar plate with colonies of *E. coli* (DH5alpha) with pCas1+Cas2
- 1x tube of LB media (**LB**)
- 1x tube of 1000x streptomycin (**Strep**)
- 1x tube of 20% arabinose (**Ara**)
- 1x tube of 0.1 M IPTG (Isopropyl β-d-1-thiogalactopyranoside) (**IPTG**)
- Distilled water
- 2x 15-mL culture tubes
- 1x 1.5-mL microcentrifuge tube
- Pipettes
- Pipette tips
- Shaking incubator set at 37˚C
- Ice-bucket

# Part 1: CRISPR Immunity cont.

Protocol:

1. Take two 15-mL culture tubes and label them **Induced** and **Uninduced**.
2. Add 5 mL sterile LB media (**LB**) into each of the two culture tubes.
3. Add 5 µL 1000x streptomycin (**Strep**) into each of the two culture tubes.
4. Add 50 µL 20% arabinose (**Ara**) and 5 µL 0.1 M IPTG (**IPTG**) to the culture tube labeled **Induced**.
5. Take one 1.5-mL microcentrifuge tube and label it **TP0** for Time Point 0.
6. Add 10 µL sterile distilled water to the tube labeled **TP0**.
7. Choose a bacterial colony on the plate provided and draw a circle around it to label the colony.
   - You will be sampling the same colony three times, so don't pick a colony that's too small.
8. Using a pipette tip, gently touch the colony with the end of the pipette tip.
9. Drop the pipette tip into the culture tube labeled **Induced**.
10. Repeat **steps 8 - 9** and drop the tip into the culture tube labeled **Uninduced**.
11. Using a third pipette tip, gently touch the same colony and swirl the tip in the water in the **TP0** tube.
12. Place the two culture tubes in a 37˚C shaking incubator overnight.
13. Place the **TP0** tube in the ice bucket at the front of the classroom. They will be frozen at -20˚C until we use them.

## Sunday 7/6 (prepared in advance)

A liquid culture is a mixed population of bacterial cells. Some cells may not have acquired spacers, and others may have acquired many. Today we will plate out some of the culture onto agar plates to get single colonies.

### Student task: Streak out *E. coli* cultures to obtain single colonies

Materials (for each pair):

- 1x culture tube with **Induced** bacterial culture
- 1x culture tube with **Uninduced** bacterial culture
- 1x LB + Streptomycin agar plate
- 2x Inoculation loops
- 2x 1.5-mL microcentrifuge tubes
- Pipette
- Pipette tips
- Ice bucket
- Incubator set at 37˚C

Protocol:

1. Take an LB + Streptomycin agar plate. Draw a line down the center and label the two halves **Induced** and **Uninduced**.
2. Take 2 µL culture from the tube labeled **Induced** and place it onto the half of the agar plate labeled **Induced**.
3. Using a sterile inoculation loop, streak out the liquid culture across that half of the agar plate.
4. Repeat **steps 2 - 3** with culture from the **Uninduced** tube and streaking on the half of the agar plate labeled **Uninduced**.
5. Leave the plate to incubate at 37˚C overnight.
6. Take two 1.5-mL microcentrifuge tubes and label them **Induced** and **Uninduced**.
7. Take 50 µL culture from the culture tube labeled **Induced** and add it to the microcentrifuge tube labeled **Induced**.
8. Repeat **step 7** with the **Uninduced** culture.
9. Place the two microcentrifuge tubes in the ice bucket at the front of the classroom. They will be frozen at -20˚C until we use them.

# Part 1: CRISPR Immunity cont.

## Monday 7/7

### Instructor preparation: Aliquot PCR reagents.

Materials:

- 2x Phusion High-Fidelity PCR Master Mix (NEB, cat. M0531S)
- 10 µM Forward Primer (5'- ATTTTGCGTTTCGTTCAGGT -3')
- 10 µM Reverse Primer (5'- TGGATGTGTTGTTTGTGTG -3')
    - Primers were ordered through IDT and reconstituted to a final concentration of 10 µM.
- Distilled water
- 40x 1.5-mL microcentrifuge tubes
- Pipettes
- Pipette tips

Protocol:

1. Take the 2x Phusion High-Fidelity PCR Master Mix, reconstituted Forward Primer, and Reverse Primer out of the freezer.
2. For each of the 10 student groups, prepare three 1.5-mL microcentrifuge tubes and label them **H2O**, **MM**, **Fwd**, and **Rev** (40 tubes total).
3. Aliquot 100 µL distilled water to the tubes labeled **H2O**.
4. Aliquot 110 µL 2x Phusion High-Fidelity PCR Master Mix to the tubes labeled **MM**.
5. Aliquot 15 µL 10 µM Forward Primer to the tubes labeled **Fwd**.
6. Aliquot 15 µL 10 µM Reverse Primer to the tubes labeled **Rev**.

# Monday 7/7

Yesterday we took samples of our induced and uninduced cultures, and today we have a plate of single colonies of each culture. We will use PCR to amplify their CRISPR arrays to determine if any spacers have been acquired. Remember a liquid culture is a mixed population. By running your PCR on the culture you can simultaneously see results from all the cells in the sample. We plated out your cultures to obtain single colonies and we will also PCR single colonies for their spacer region. By performing PCR on single colonies, we won't get a mixture of different spacers but we can see the acquired spacer of that one colony. We can send those PCR products from single colonies to sequencing, and see the sequence of the spacer acquired by this colony.

## Student task: Set up and run a PCR

Materials (for each pair):

- 1x 1.5-mL microcentrifuge tube **TP0**
- 1x 1.5-mL microcentrifuge tube with **Induced** culture
- 1x 1.5-mL microcentrifuge tube with **Uninduced** culture
- 1x agar plate with colonies of **Induced** and **Uninduced** cultures
- 1x tube of Water (**H2O**)
- 1x tube of 2x Phusion High-Fidelity PCR Master Mix (**MM**)
- 1x tube of 10 µM Forward Primer (**Fwd**)
- 1x tube of 10 µM Reverse Primer (**Rev**)
- 1x 1.5-mL microcentrifuge tube
- 7x 0.2-mL PCR tubes
- Pipettes
- Pipette tips
- Thermal cycler

# Part 1: CRISPR Immunity cont.

Protocol:

1. Gather your **streaked agar plate**, and microcentrifuge tubes **TP0**, **Induced**, and **Uninduced**.
2. Take a 1.5-mL microcentrifuge tube and label it **MMP** for PCR master mix with primers.
3. Follow the table below and add reagents to the tube labeled **MMP**. The total volume of the master mix is 400 µL.

| Volume (µL) | Reagent | Final Concentration |
|---|---|---|
| 80 µL | Water (**H2O**) | |
| 100 µL | 2x Phusion High-Fidelity PCR Master Mix (**MM**) | 1x |
| 10 µL | Forward primer (10 µM) **Fwd** | 0.5 µM |
| 10 µL | Reverse primer (10 µM) **Rev** | 0.5 µM |

4. Take seven PCR tubes and label them **TP0**, **I**, **U**, **IC1**, **IC2**, **UC1**, and **UC2**.
5. Add 25 µL PCR master mix with pimers (**MMP**) to each of the seven PCR tubes.
6. Take 2µL liquid from the microcentrifuge tube labeled **TP0** and add it to the PCR tube labeled **TP0**.
7. Repeat **step 6** with cultures from microcentrifuge tubes labeled **Induced** and **Uninduced**, and add it to the PCR tubes labeled **I** and **U** respectively.
8. Choose two bacterial colonies on the **Induced** half of the plate and two colonies on the **Uninduced** half. Draw a circle around them to label the colonies.
9. Using a pipette tip, gently touch one of the colonies on the **Induced** half with the end of the pipette tip.
10. Swirl the pipette tip in the PCR tube labeled **IC1** (induced colony 1).
11. Repeat **steps 9 - 10**, with the second colony on the **Induced** half of the plate and swirl the tip in the PCR tube labeled **IC2** (induced colony 2).
12. Repeat **steps 9 - 11**, with the two colonies on the **Uninduced** half of the plate, swirling them in the PCR tubes **UC1** (uninduced colony 1) and **UC2** (uninduced colony 2).
13. Cap all PCR tubes.

14. Place the PCR tubes in the thermal cycler and run the following program.

| Step | Temp, Time | |
|---|---|---|
| 1 Initial denature | 98°C, 3 min | 1x |
| 2 Denature | 98°C, 10 s | 35x cycles |
| 3 Anneal | 61°C, 30 s | |
| 4 Extension | 72°C, 30 s | |
| 5 Final extension | 72°C, 3 min | 1x |
| 6 Hold | 10°C, infinity | |

15. Once the PCR has finished, proceed to running the samples on a gel.

- You can freeze and store your samples at -20˚C if you are running the gel on another day.

## Instructor preparation: Aliquot reagents for agarose gel

Materials:

- 1x TAE (Often purchasable as liquid concentrate or powder. Follow manufacturer's instructions to prepare 1X TAE Buffer.)
- 10,000x GelRed Nucleic Acid Stain (MilliporeSigma, cat. SCT123)
- 6x Purple Gel Loading Dye (NEB, cat. B7025S) Comes with most NEB enzymes
- Quick-Load Purple 100 bp DNA Ladder (NEB, cat. N0551S)
- 30x 1.5-mL microcentrifuge tubes
- Pipettes
- Pipette tips

Protocol:

1. Prepare 1x TAE Buffer.
2. Take out the 10,000x GelRed Nucleic Acid Stain.
3. Take the 6x Purple Gel Loading Dye and Quick-Load Purple 100 bp DNA Ladder out of the freezer.
4. For each of the 10 student groups, prepare three 1.5-mL microcentrifuge tubes and label them **LD**, **GR**, and **MWR** (30 tubes total).
5. Aliquot 40 µL of 6x Purple Gel Loading Dye to the tubes labeled **LD**.
6. Aliquot 5 µL 10,000x GelRed Nucleic Acid Stain to the tubes labeled **GR**.
7. Aliquot 15 µL Quick-Load Purple 100 bp DNA Ladder to the tubes labeled **MWR**.

# Part 1: CRISPR Immunity cont.

## Student task: Running agarose gel to analyse the PCR

Materials (for each pair):

- Agarose - at the weigh station
- 1x TAE - at the front of the class
- 1x tube of 6x Loading Dye (**LD**)
- 1x tube of 10,000x GelRed Nucleic Acid Stain (**GR**)
- 1x tube of 100 bp DNA ladder (**MWR**)
- Gel electrophoresis chamber
- 1x Gel tray
- 1x 12-well comb
- 1x 150-mL conical flask
- 1x 50-mL measuring cylinder
- Microwave
- Power pack
- Plastic wrap
- Pipettes
- Pipette tips

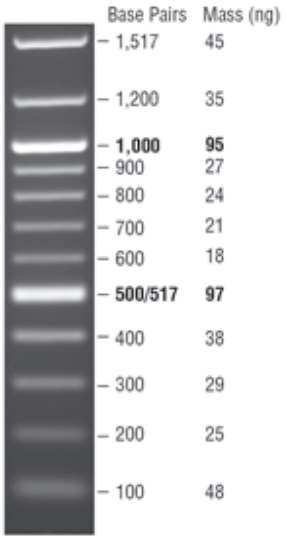
Protocol:

1. While the thermal cycler program is running, pour a 1% agarose gel.
2. Prepare a gel mold with a 12-well comb
3. Weigh out 0.5 g agarose into a 150-mL conical flask.
4. Measure out 50 mL 1x TAE buffer in a measuring cylinder and add it to the flask with agarose.
5. Cover the top of the flask with plastic wrap and microwave until it just starts to boil. Do not let it boil over.
6. Repeat **step 5** until the agarose is fully dissolved.
7. Allow the flask to cool down to about 60˚C.
8. Add 5 µL GelRed Nucleic Acid Stain (**GR**) to the cooled flask and swirl to mix.
9. Pour the mixture into the gel mold.

10. Leave the gel to cool and solidify.
11. Place the 1% TAE agarose gel into the electrophoresis tank and fill the tank with 1x TAE buffer.
12. Once the PCR has finished, add 5 µL 6x loading dye (**LD**) to each of the seven PCR tubes.
13. Using a new pipet tip for each sample, load 15 µL of each of the following samples

    1. Quick-Load Purple 100 bp DNA Ladder (**MWR**)
    2. Tube **TP0**
    3. Tube **I** (Induced culture)
    4. Tube **U** (Uninduced culture)
    5. Tube **IC1** (Induced colony 1)
    6. Tube **IC2** (Induced colony 2)
    7. Tube **UC1** (Uninduced colony 1)
    8. Tube **UC2** (Uninduced colony 2)

14. Place the lid on the electrophoresis tank.
15. Run the gel at 120V for 20 minutes.
16. Visualize the gel under UV light/blue light.
17. Examine your gel and celebrate! You're done!




Use this as a reference for how the Quick-Load Purple100 bp DNA Ladder runs on a 1% agarose gel.

Additional task:

Give the instructor one PCR product with a successful amplification of the parent locus (from one of the two uninduced colonies) and one PCR product with newly acquired spacers (the ones that appear shifted up with respect to the parent band on the gel in your induced colonies). We will send these samples to the sequencing facility along with the Forward Primer (Fwd). The sequencing facility will do a PCR cleanup and then perform Sanger sequencing using the primer provided.

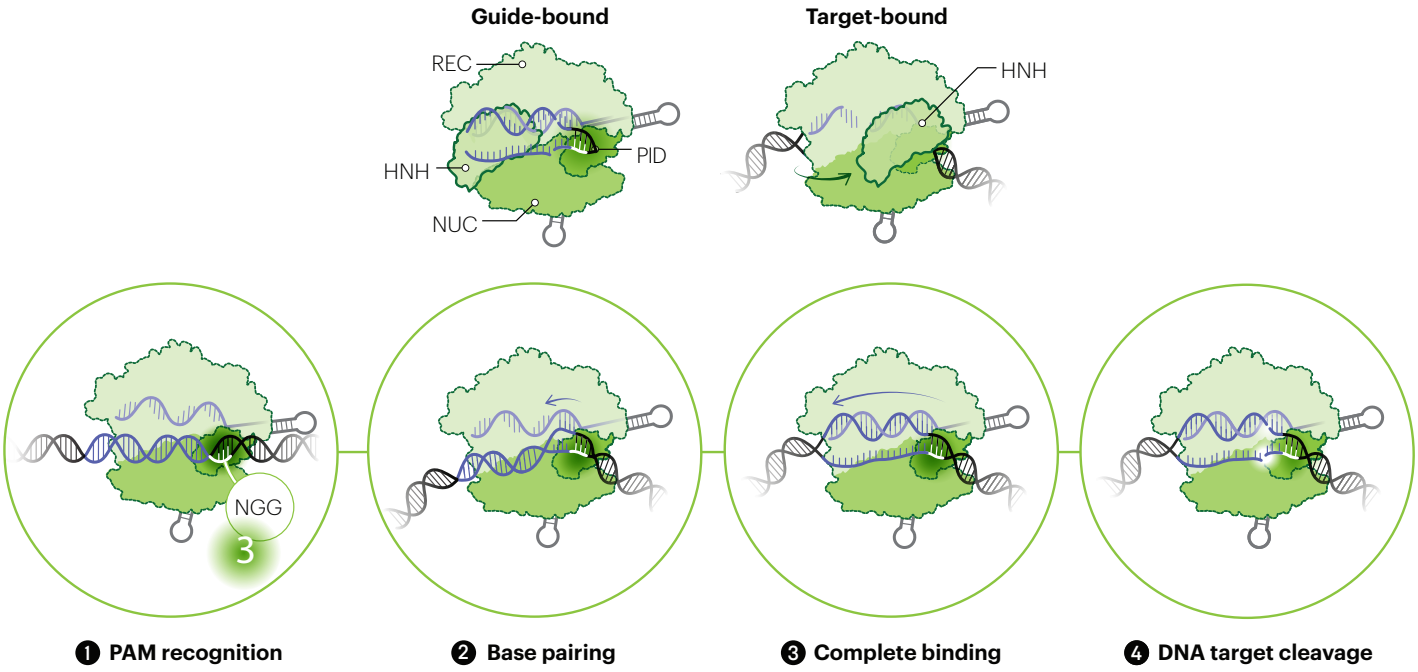
Once the sequences come in:

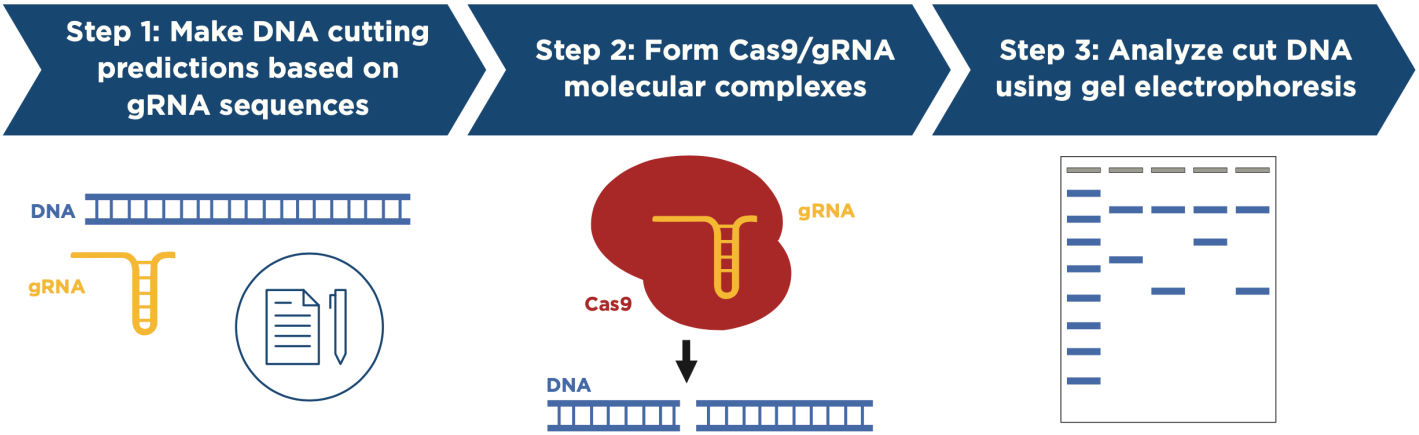
You will compare the sequencing results. By analyzing the sequences, you will determine where the spacers came from in your bacterial colonies. We will guide you through opening/viewing a sample sequencing file and how to determine the origin of a spacer sequence.

**Instructors notes:** Feel free to use any software you are comfortable teaching such as Benchling or SnapGene.

# Part 2: *In Vitro* Cleavage Assay using Cas9

Today we will be leveraging Cas9 to cut DNA in an experiment known as an *in vitro* cleavage assay. This experiment will show how we can precisely direct the Cas9 enzyme to cut DNA at a chosen location simply by altering its guide RNA. By combining this targeted cutting ability with the natural DNA repair mechanisms found in living cells, scientists have been able to use Cas9 to edit the genomes of a wide range of organisms—including bacteria, plants, animals, and even humans.



## Tuesday 7/8

### Instructors preparation: Aliquot reagents for *in vitro* cleavage assay

Materials (for 8 groups):

- miniPCR Chopped! Kit
  - 1x tube of Nuclease-Free Water
  - 1x tube of Reaction Buffer
  - 1x tube of 5x Cas9 Nuclease Concentrate
  - 1x tube of gRNA1
  - 1x tube of gRNA2
  - 1x tube of DNA Sample
  - 1x tube of Proteinase K Solution (green solution as it also contains gel loading dye)
  - 1x tube of Fast DNA Ladder 3
- 56x 0.5-mL microcentrifuge tubes
- Ice bucket + Ice
- Pipettes
- Pipette tips

Protocol:

1. Take the following reagents out of the freezer.
   - Nuclease-Free Water
   - Reaction Buffer
   - gRNA1
   - gRNA2
   - DNA Sample
   - Proteinase K Solution
   - Fast DNA Ladder
2. Thaw all the tubes at room temperature.
3. Take out the 5x Cas9 Nuclease Concentrate out of the freezer onto ice. The nuclease will remain a liquid at -20˚C, therefore, does not need to be thawed.
   - Cas9 nuclease must be kept in the freezer or on ice at all times!
4. Add 160 µL Nuclease-Free Water to the 5x Cas9 Nuclease Concentrate tube. This will yield 200 µL of 1x Cas9 Nuclease solution.
5. For each of the 8 student groups, prepare seven 0.5-mL microcentrifuge tubes and label them **Water**, **Rxn Buf**, **gRNA1**, **gRNA2**, **DNA**, **PK**, and **Cas9** (56 tubes total).

# Part 2: *In Vitro* Cleavage Assay using Cas9 cont.

6. Aliquot 20 µL Nuclease-Free Water to each of the eight tubes labeled **Water**.
7. Aliquot 25 µL Reaction Buffer to each of the eight tubes labeled **Rxn Buf**.
8. Aliquot 10 µL gRNA1 to each of the eight tubes labeled **gRNA1**.
9. Aliquot 10 µL gRNA2 to each of the eight tubes labeled **gRNA2**.
10. Aliquot 25 µL DNA Sample to each of the eight tubes labeled **DNA**.
11. Aliquot 20 µL Proteinase K Solution to each of the eight tubes labeled **PK**.
12. Aliquot 20 µL 1x Cas9 Nuclease Solution to each of the eight tubes labeled **Cas9**. Store on ice.
13. Store all aliquots in a -20˚C freezer until class.

## Tuesday 7/8

Today we will perform the *in vitro* cleavage assay. First, we will form the ribonucleoprotein (RNP) complex composed of the gRNA and Cas9 protein. Second, we will introduce DNA that contains a sequence that matches the gRNA. Third, we will add Proteinase K to inactivate the Cas9 enzyme. And fourth, we will run the reaction on an agarose gel, to visualize the fragments of DNA that result from cleavage by the RNP.

First, a note on working with RNA. RNA is very unstable, and easily digested by the RNase enzymes on our skin and in our saliva. Wipe down everything on your workstation with 70% ethanol (RNase Away if you have any) to ensure no contamination. Try not to breathe directly onto or speak over open sample tubes.

### Student task: Set up *in vitro* cleavage assay

Materials:

- 1x tube of Nuclease-Free Water (**Water**)
- 1x tube of Reaction Buffer (**Rxn Buf**)
- 1x tube of gRNA1 (**gRNA1**)
- 1x tube of gRNA2 (**gRNA2**)
- 1x tube of DNA Sample (**DNA**)
- 1x tube of Proteinase K (**PK**)

- 1x tube of 1x Cas9 Nuclease (**Cas9**) kept on ice at all times!
- 4x 0.5-mL microcentrifuge tubes
- Ice bucket + ice
- Vortex mixer
- Microcentrifuge
- Dry bath / Water bath / Incubator set to 37˚C
- Stopwatch
- Pipettes
- Pipette tips

Protocols:

1. Take four 0.5-mL microcentrifuge tubes and label them **A - D**.
2. Follow the table below and add reagents to each of the tubes.

| | **Tube A** | **Tube B** | **Tube C** | **Tube D** |
|---|---|---|---|---|
| | Control 1<br>No gRNA<br>No Cas9 | Control 2<br>No gRNA | gRNA 1 | gRNA 2 |
| Nuclease-Free Water | 10 | 5 | - | - |
| Reaction Buffer | 5 | 5 | 5 | 5 |
| Cas9 Nuclease | - | 5 | 5 | 5 |
| gRNA1 | - | - | 5 | - |
| gRNA2 | - | - | - | 5 |
| Total volume | 15 | 15 | 15 | 15 |

3. Vortex the tubes for 5 seconds to mix.
4. Spin the tubes for 2 seconds in a microcentrifuge to bring all the liquid volume to the bottom of the tube.
   - If no microcentrifuge is available, you can flick your wrist to shake the liquid down to the bottom of the tube.
5. Incubate the tubes at room temperature for 10 minutes.
6. Add 5 µL of DNA Sample (**DNA**) to each of the tubes.
7. Vortex the tubes for 5 seconds to mix.
8. Spin the tubes for 2 seconds in a microcentrifuge to bring all the liquid volume to the bottom of the tube.
   - If no microcentrifuge is available, you can flick your wrist to shake the liquid down to the bottom of the tube.
9. Place the tubes into a water bath set at 37˚C and incubate for 15 minutes.

# Part 2: *In Vitro* Cleavage Assay using Cas9 cont.

10. Take the tubes out of the water bath.
11. Add 4 µL Proteinase K (**PK**) to each of the tubes.
    - The Proteinase K Solution is green because it also contains gel loading dye for running the sample on the gel.
12. Vortex the tubes for 5 seconds to mix.
13. Spin the tubes for 2 seconds in a microcentrifuge to bring all the liquid volume to the bottom of the tube.
    - If no microcentrifuge is available, you can flick your wrist to shake the liquid down to the bottom of the tube.
14. Place the tubes into a water bath set at 37˚C and incubate for 10 minutes.
15. Take the tubes out of the water bath and proceed to running them on a gel.
    - You can freeze and store your samples at -20˚C if you are running the gel on another day.

## Instructor preparation: Aliquot reagents for agarose gel

Materials:

- 1x tube Fast DNA Ladder 3 (provided in the kit)
- 1x TAE (Often purchasable as liquid concentrate or powder. Follow manufacturer's instructions to prepare 1X TAE Buffer.)
- 10,000x GelRed Nucleic Acid Stain (MilliporeSigma, cat. SCT123)
- 16x 1.5-mL microcentrifuge tubes
- Pipettes
- Pipette tips

Protocol:

1. Prepare 1x TAE Buffer.
2. Take out the 10,000x GelRed Nucleic Acid Stain.
3. Take the Fast DNA Ladder 3 out of the freezer.
4. For each of the 8 student groups, prepare two 1.5-mL microcentrifuge tubes and label them **GR** and **MWR** (16 tubes total).
5. Aliquot 5 µL 10,000x GelRed Nucleic Acid Stain to the tubes labeled **GR**.
6. Aliquot 15 µL Fast DNA Ladder 3 to the tubes labeled **MWR**.

## Student task: Running agarose gel to analyse the cleavage assay

Materials:

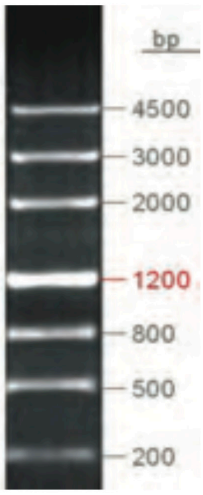
- Agarose - at the weigh station
- 1x TAE - at the front of the class
- 1x tube of 10,000x GelRed Nucleic Acid Stain (**GR**)
- 1x tube of Fast DNA Ladder 3 (**MWR**)
- Gel electrophoresis chamber
- 1x Gel tray
- 1x 12-well comb
- 1x 150-mL conical flask
- 1x 50-mL measuring cylinder
- Microwave
- Power pack
- Plastic wrap
- Pipettes
- Pipette tips

Protocol:

1. While the reactions are incubating, pour a 1% agarose gel.
2. Prepare a gel mold with a 12-well comb
3. Weigh out 0.5 g agarose into a flask.
4. Measure out 50 mL 1x TAE buffer in a measuring cylinder and add it to the flask with agarose.
5. Cover the top of the flask with plastic wrap and microwave until it just starts to boil. Do not let it boil over.
6. Repeat **step 5** until the agarose is fully dissolved,
7. Allow the flask to cool down to about 60˚C.
8. Add 5 µL GelRed Nucleic Acid Stain (**GR**) to the cooled flask and swirl to mix.
9. Pour the mixture into the gel mold.
10. Leave the gel to cool and solidify.
11. Place the 1% TAE agarose gel into the electrophoresis tank and fill the tank with 1x TAE buffer.
12. Once the Proteinase K digestion is finished, gather the tubes.
13. Using a new pipet tip for each sample, load 15 µL of each of the following samples
    1 Fast DNA Ladder 3 (**MWR**)
    2 Tube **A**
    3 Tube **B**
    4 Tube **C**
    5 Tube **D**

# Part 2: *In Vitro* Cleavage Assay using Cas9 cont.

14. Place the lid on the electrophoresis tank.
15. Run the gel at 120V for 20 minutes.
16. Visualize the gel under UV light/blue light.
17. Examine your gel and celebrate! You're done!




Fast DNA Ladder 3.

Use this as a reference for how the Fast DNA Ladder 3 runs on a 1% agarose gel.

Notes

# Part 3: *In Vivo* Genome Editing of *E. coli* using Cas9

We will now move from cutting DNA in a test tube (*in vitro*) to cutting DNA inside living cells (*in vivo*). Cleaving DNA within a cell allows researchers to introduce new genetic sequences, which can result in the organism acquiring or losing specific traits.

This section will be split into two following each of the two kits from LabAids and BioRad.

LabAids: The Power of CRISPR

In this section, we will begin with a fluorescent *E. coli* strain, which has the red fluorescent protein (RFP) constitutively expressed in the genome. These cells glow red/orange. The goal of the *in vivo* genome editing is to insert the green fluorescent protein (GFP) gene into the *E. coli* genome at the site of the RFP gene. If successful, the cells should switch from appearing red to appearing green.

The *E. coli* already has the plasmids containing all the components required for the gene editing inside. The key components needed for the editing are the following:

- Cas9 nuclease (under the tet promoter)
- RFP-targeting sgRNA (under the araBAD promoter)
- Lambda red recombineering genes (under the araBAD promoter)
- Donor GFP cassette with homology

The lambda red recombineering genes encode for proteins that the *E. coli* uses to repair DNA after the Cas9 nuclease cuts it. *E. coli* do not have robust repair proteins, so these need to be included for more efficient gene editing success.

## Saturday 7/5

This is a classroom sized kit and contains material for 8 groups to conduct the experiments. For more detailed instructions please refer to the "The Power of CRISPR" Teacher's Guide. The Teacher's Guide is provided with the kit, and the digital version is also available on the workshop drive (USB and cloud).

### **Instructor preparation:** Preparing LB agar plates.

Materials:

- 1x bottle of **ReadyPour LB Agar**
- 1x tube of **Component A: Ampicillin** (for dishes) (contains: ampicillin, kanamycin, chloramphenicol) (powder form)
- 1x tube of **Component B: Glucose** (for dishes) (liquid form)
- 32x Sterile petri dish plates
- 1x 10-mL serological pipette
- 1x serological pipette pump
- Microwave
- Heat-resistant safety gloves
- Sealable plastic bag
- Pipettes
- Pipette tips

Protocol:

1. Take the **ReadyPour LB Agar** out of the refrigerator. Take **Component A (Amp)**, and **Component B (glucose)** out of the freezer.
2. Allow the **ReadyPour LB Agar**, **Component A**, and **Component B** warm up to room temperature.
3. Loosen the cap of the **ReadyPour LB Agar** bottle. Do not remove the cap.
4. Microwave the **ReadyPour LB Agar** bottle until the agar is completely dissolved.
   - Microwave on high for 1 minute.
   - Wearing heat-resistant gloves, remove the bottle from the microwave and close the cap.
   - Squeeze the bottle and vigorously shake it to break up the agar into small chunks.
   - Microwave again until the solution starts to bubble. Do not let it boil over.
   - Remove the bottle from the microwave and swirl to mix the agar and dissipate the heat.
   - Repeat with short microwave intervals until the agar is completely dissolved.
   - The molten **LB Agar** solution should be clear and free of small particles.
5. Leave the **ReadyPour LB Agar** on the benchtop to cool down to 60˚C. Swirl intermittently to dissipate the heat.
6. While the molten **LB Agar** is cooling, prepare the petri-dish plates. Take the petri-dish plates out of the sleeve and line them up. Do not open individual dishes.
7. Once the molten **LB Agar** has cooled down, add all of **Component A (Amp)** and **Component B (glucose)** to the cooled medium.
   - Be sure the molten **LB Agar** has cooled down enough before proceeding, as the reagents will degrade at higher temperatures.
   - You can transfer 1 mL of the molten **LB Agar** into the **Component A (Amp)** tube, mix, and transfer it back into the **LB Agar** bottle. Repeat this at least twice to thoroughly rinse out the **Component A (Amp)** tube.
8. Recap the bottle and swirl to mix the reagents.
9. Pour at least 32 plates. Tilt the lid of a petri-dish plate open and transfer 10 mL of the **LB Agar** into the plate. Immediately replace the lid, and gently swirl if necessary to distribute the agar evenly.
   - **Highly Recommend:** Use the 10-mL serological pipette provided in the kit to properly measure out the volume of **LB Agar**. If hand poured, you will likely over pour and not have enough plates at the end. They provide 50 petri-dish plates and enough **LB Agar** to pour 36 dishes with 10 mL, resulting in 4 extra plates in case.
10. Leave the plates to cool and solidify. They can be left at room temperature overnight.
11. Stack the dried plates and place them in a sealable plastic bag.
12. Store the plates lid-side down in a 4˚C refrigerator for later use.

# Part 3: *In Vivo* Genome Editing of *E. coli* using Cas9 cont.

## Sunday 7/6

### Instructor preparation: Streak out the bacteria from the BactoBeads.

Materials:

- 1x bottle of Rehydration Media
- 1x vial of **Experimental** CRISPR BactoBeads
- 1x vial of **Control** CRISPR BactoBeads
- 16x LB + Amp + Glucose agar plates (from yesterday's Instructor Preparation)
- 2x sterile inoculation loops
- 2x sterile graduated transfer pipettes
- Pipettes
- Pipette tips
- Incubator set at 37˚C

Protocol:

1. Take the Rehydration Media out of the freezer. Take **Experimental** CRISPR BactoBeads and **Control** CRISPR BactoBeads out of the refrigerator.
2. Thaw the rehydration media and allow the two CRISPR BactoBeads to warm up to room temperature.
3. Take the LB + Amp + Glucose agar plates and label eight plates **Experimental** and eight plates **Control**.
4. Transfer an **Experimental** CRISPR BactoBead to an LB agar plate labeled **Experimental**.
   - Using a sterile inoculation loop, pick up a single **Experimental** CRISPR BactoBead from the tube.
   - Tilt open the lid of an LB agar plate labeled **Experimental**.
   - Transfer the bead onto the agar, and replace the lid.
5. Repeat **step 4** for the seven remaining **Experimental** CRISPR BactoBeads and transfer them onto LB agar plates labeled **Experimental**.
   - Save the inoculation loop for **Step 8**.
6. Rehydrate the **Experimental** CRISPR BactoBead on the plates with Rehydration Media.
   - Add 1 drop of Rehydration Media onto the **Experimental** CRISPR BactoBead using a transfer pipette.

- Alternatively, you can transfer 100 µL Rehydration Media using a pipette.

7. Repeat **step 6** to add Rehydration Media to the remaining seven **Experimental** CRISPR BactoBeads in the **Experimental** LB agar plates.
8. Streak out the **Experimental** bacteria using the inoculation loop from **step 4**.
   - Streak the inoculation loop gently back and forth through the rehydrated BactoBead across the surface of the agar.
   - Caution to not jab or poke the agar with the loop.
9. Repeat **steps 4 - 8** to transfer, rehydrate, and streak out the **Control** CRISPR BactoBeads onto the **Control** LB agar plates.
10. Turn the plates lid-side down, and leave them to incubate at 37˚C overnight.
    - Alternatively, the plates can be incubated at room temperature for 3 - 4 days.

## Monday 7/7

### Instructor preparation: Prepare liquid LB and aliquot for each group.

Materials:

- 1x bottle of sterile **Liquid LB media**
- 1x tube of **Component C: Ampicillin** (for liquid LB)
- 1x tube of **Component D: Chloramphenicol** (for liquid LB)
- 1x tube of **Component E: Glucose** (for liquid LB)
- 1x sterile graduated transfer pipette
- 8x sterile 15-mL conical tubes
- Pipettes
- Pipette tips

Protocol:

1. Take the **Liquid LB media** out of the refrigerator. Take **Component C (Amp)**, **Component D (Cam)**, and **Component E (glucose)** out of the freezer.
2. Allow the **Liquid LB media**, **Component C (Amp)**, **Component D (Cam)**, and **Component E (glucose)** to warm up to room temperature.
3. Add all of **Component C (Amp)**, **Component D (Cam)**, and **Component E (glucose)** to the **Liquid LB media**.
4. Swirl the **Liquid LB media** bottle to mix the reagents. Do not shake.
5. For each of the 8 student groups, prepare one 15-mL conical tube and label them **LB** (8 tubes total).
6. Aliquot the **Liquid LB media** into the eight tubes labeled **LB**. Roughly 7 mL per tube.
7. Store the **LB** tubes in a 4˚C refrigerator until the start of class.

# Part 3: *In Vivo* Genome Editing of *E. coli* using Cas9 cont.

Today we will be inoculating colonies from the **Experimental** and **Control** agar plates to prepare for tomorrow's induction.

## Student task: Inoculating the Experimental and Control bacteria colonies.

Materials (for each group):

- 1x agar plate labeled **Experimental**
- 1x agar plate labeled **Control**
- 1x tube of Liquid LB + Amp + Cam + Glucose media (**LB**)
- 2x 15-mL culture tubes
- 2x sterile inoculation loops
- 2x sterile graduated transfer pipette
- Pipettes
- Pipette tips
- Shaking incubator set at 37˚C

Protocol:

1. Take two 15-mL culture tubes and label one **Experimental** and the other **Control**.
2. Using the graduated transfer pipette, transfer 1 mL Liquid LB media (**LB**) into each of the two culture tubes.
    - Alternatively, use a P1000 and transfer 1 mL.
3. Choose a bacterial colony on the **Experimental** plate and draw a circle around it to label the colony.
4. Using a sterile inoculation loop, touch the colony on the **Experimental** plate
5. Place the loop with the bacteria into the culture tube labeled **Experimental**, and mix it into the liquid LB by swirling the loop in the liquid LB. Make sure that there are no visible clumps of bacteria left on the loop.
6. Cap the culture tube. The cap should stay on but be able to move up and down.
7. Using a second sterile inoculation loop, repeat **steps 3 - 6** for the **Control** plate, adding the bacteria to the culture tube labeled **Control**.
8. Place the two culture tubes in a 37˚C shaking incubator overnight.
    - Alternatively, store the culture tubes at room temperature for 24 hours.

## Tuesday 7/7

### Instructor preparation: Prepare inducer solution.

Materials:

- 1x tube of **Component F: Anhydrotetracycline** (inducer)
- 1x bottle of **Component G: Arabinose** (inducer)
- 2x sterile graduated transfer pipettes
- 8x sterile 1.5-mL microcentrifuge tubes
- Pipettes
- Pipette tips

Protocol:

1. Take **Component F (Anhydrotetracycline)** and **Component G (Arabinose)** out of the freezer.
2. Allow the **Component F (Anhydrotetracycline)** and **Component G (Arabinose)** to warm up to room temperature.
3. Transfer all of **Component F (Anhydrotetracycline)** to **Component G (Arabinose)**.
4. Swirl the bottle to mix the two solutions to make the Inducer solution.
5. For each of the 8 student groups, prepare one 1.5-mL microcentrifuge tube and label them **Inducer** (8 tubes total).
6. Aliquot 1 mL Inducer solution to the tubes labeled **Inducer**. You can use a sterile transfer pipette or a P1000.
7. Store the **Inducer** tubes in a 4˚C refrigerator and keep them in the dark until the start of class.

Today we will be inducing the bacterial cultures. The bacteria have plasmids to express the Cas9 nuclease, the sgRNA, and the lambda red recombineering system. In the **Experimental** bacteria the sgRNA targets the RFP gene. In the **Control** bacteria the sgRNA does not target the RFP gene or any other sequence in the bacterial genome. In the **Experimental** bacteria the expression of these genes will allow for the integration of the GFP cassette into the genome. Following induction, the bacteria will be plated on LB agar plates and evaluated by fluorescence the following day for proper integration.

# Part 3: *In Vivo* Genome Editing of *E. coli* using Cas9 cont.

## Student task: Induce your culture and streak it out.

Materials (for each group):

1x tube of Inducer solution (**Inducer**)

- 1x culture tube with **Experimental** bacterial culture
- 1x culture tube with **Control** bacterial culture
- 2x LB + Amp + Glucose agar plates
- 2x sterile inoculation loops
- 2x sterile graduated transfer pipette
- Pipettes
- Pipette tips
- Shaking incubator set at 37˚C
- Incubator set at 37˚C

Protocol:

1. Retrieve your overnight culture from the front of the room.
2. Using a sterile transfer pipette, draw up the inducer solution (**Inducer**).
3. Remove the cap of the **Control** culture tube and add 1 drop of inducer solution (**Inducer**) to the tube.
    - Alternatively, add 100 µL of inducer solution (**Inducer**) using a pipette.
4. Cap the culture tube. The cap should stay on but be able to move up and down.
5. With a new transfer pipette, repeat **steps 2 - 4** and add inducer solution (**Inducer**) to the **Experimental** culture tube.
6. Place the two culture tubes in a 37˚C shaking incubator and incubate for 1 hour.
    - Alternatively, store the culture tubes at room temperature for 24 hours.
7. Take two LB + Amp + Glucose agar plates and label one **Experimental Induced** and the other **Control Induced**.
8. After 1 hour of incubation, use a transfer pipette to draw up a small amount of culture in the **Control** culture tube.
9. Tilt open the **Control Induced** plate and add a drop of the **Control** culture onto the agar.

- Alternatively, you can take 100 µL using a pipette.

10. Using a sterile inoculation loop gently spread the liquid droplet across the agar dish.
11. Using a new transfer pipette and inoculation loop, **repeat steps 8 - 10** for the **Experimental** culture tube and streak out on the **Experimental Induced** plate.
12. Turn the plates lid-side down, and leave them to incubate at 37˚C overnight.
    - Alternatively, store the plates at room temperature for 72 hours.

## Wednesday 7/9

Today, you will check the fluorescence of your colonies. Only the bacteria with the sgRNA targeting the RFP gene should have undergone GFP cassette integration and should appear green.

### Student task: Check fluorescence

Materials (for each group):

- 1x long-wave UV flashlight
- 1x UV viewing box with a black paper insert
- 1x agar plate labeled **Experimental** (Instructor prep)
- 1x agar plate labeled **Control** (Instructor prep)
- 1x agar plate labeled **Experimental Induced**
- 1x agar plate labeled **Control Induced**

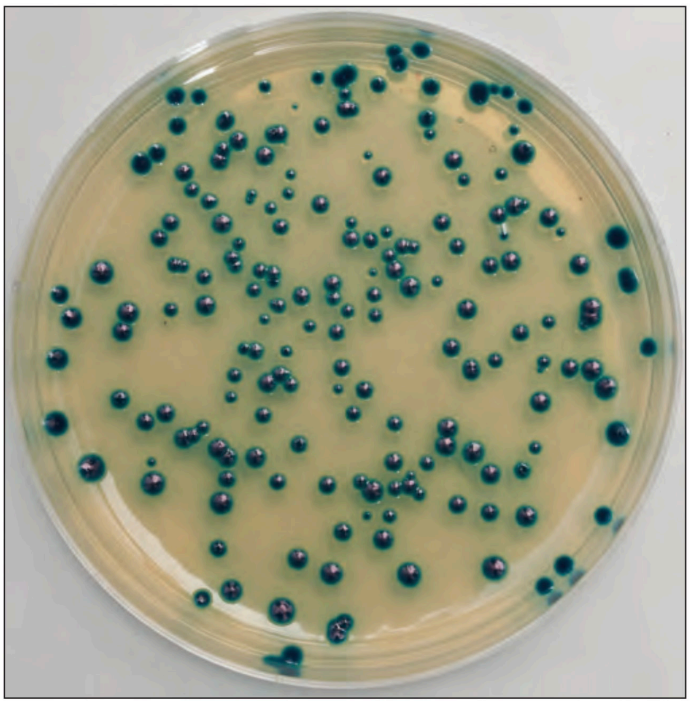
Protocol:

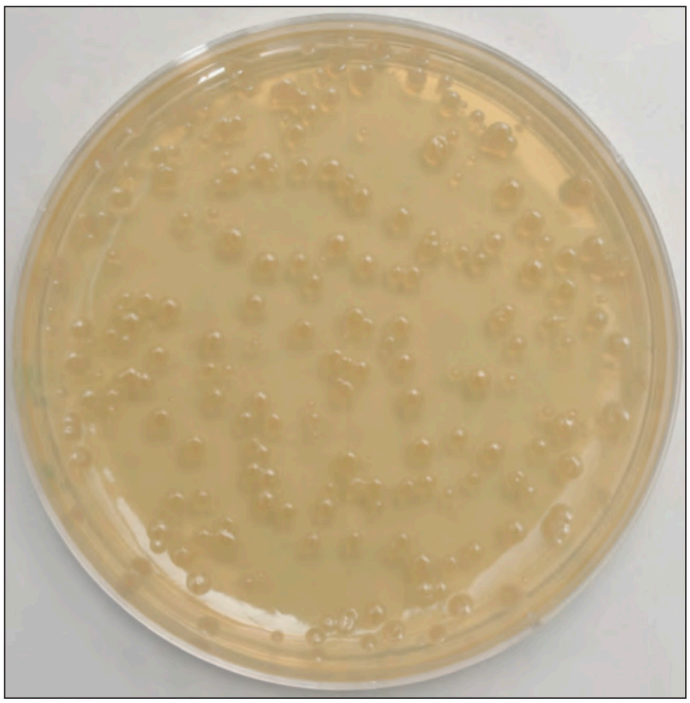
1. Fold and shape the UV viewing box with the orange film facing upwards..
2. Place the black sheet of paper on the bottom of the box. This helps for contrast.
3. Place your agar plates into the UV viewing box with the bacteria facing up.
4. Shine the UV flashlight through the side opening of the box, and observe the color of the bacteria through the orange film.
5. Take a photo and celebrate! You're done!

# Part 3: *In Vivo* Genome Editing of *E. coli* using Cas9 cont.

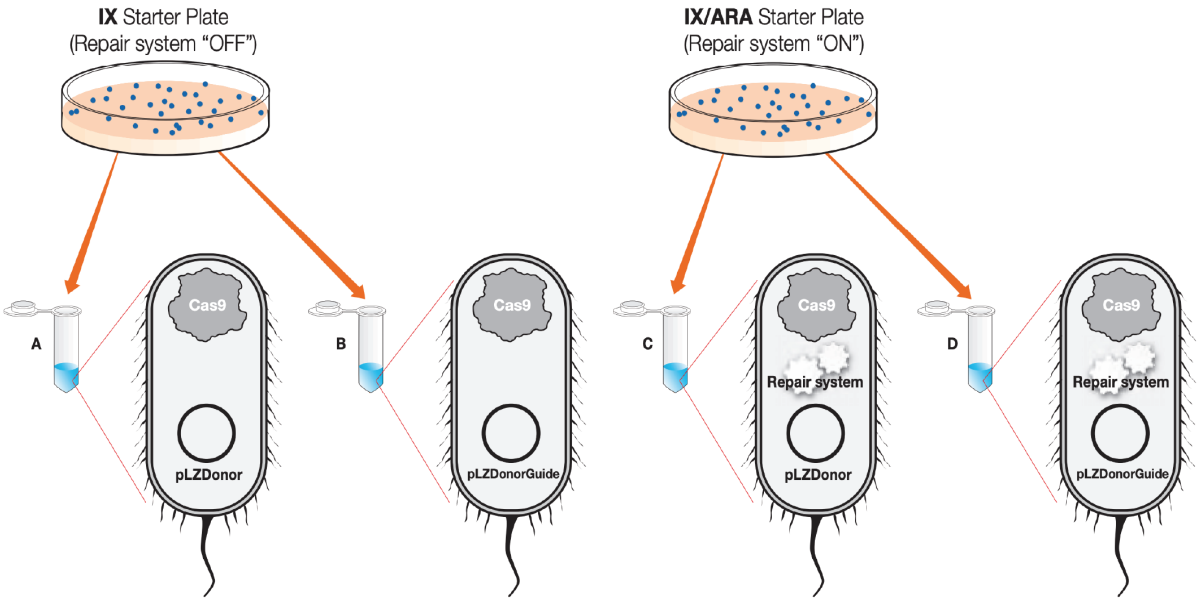
BioRad: Out of the Blue CRISPR

In this section, we will begin with an *E. coli* strain engineered to express the Cas9 nuclease and that harbors a plasmid containing genes for homology directed repair. The repair genes are under an arabinose inducible promoter. These cells express the lacZ gene, encoding for ß-galactosidase (ß-gal), which allows the *E. coli* to hydrolyze lactose. ß-gal can also hydrolyze a lactose analog, X-Gal, leading to the formation of a blue pigment. A non-functional ß-gal cannot hydrolyze X-Gal and doesn't produce the blue pigment, remaining white, hence, also known as the blue/white screening method.





The goal of the *in vivo* genome editing is to insert a stop codon into the lacZ gene. If successful, the bacteria should have an impaired lacZ gene and remain white. We will be transforming a second plasmid into the *E. coli*. There are two plasmids pLZDonor and pLZDonorGuide. The pLZDonor contains the donor DNA template sequence with a stop codon in the lacZ gene. The pLZDonorGuide additionally contains the sgRNA that targets the lacZ gene in the bacterial genome.




## Sunday 7/6

This is a classroom sized kit and contains material for 8 groups to conduct the experiments. For more detailed instructions, please refer to the BioRad Out of the Blue CRISPR kit Instructors Guide.

### Instructor preparation: Pour LB agar plates and rehydrate the lyophilized *E. coli.*

Materials:

- 1x vial of ***E. coli* HB101-pBRKan** (lyophilized)
- 1x vial of **KIX Mix** (Kanamycin, IPTG, X-Gal
- 1x vial of **Arabinose** (lyophilized)
- 1x vial of **Spectinomycin powder**
- 1x bottle of **LB agar** powder
- 1x **LB broth** capsule
- Sterile distilled water
- 56x 60 mm petri-dish plates
- 2x 1-L conical flasks
- 1x 100-mL bottle
- 1x 1-L measuring cylinder
- 1x 50-mL measuring cylinder
- Weighing scale
- Microwave
- Heat-resistant safety gloves
- Sealable plastic bag
- Pipettes
- Pipette tips
- Incubator set at 37˚C

# Part 3: *In Vivo* Genome Editing of *E. coli* using Cas9 cont.

Protocol:

1. Take out the **Arabinose** vial, **Spectinomycin** vial, and **KIX Mix** vial out of the freezer.
2. Take 2 1-L conical flasks and label them **KIX** and **KIX/SPT**.
3. Add 3 mL sterile deionized water to the vial of **Arabinose**. Vortex to mix. Briefly vortex every few minutes to fully dissolve. **Arabinose** may take more than 10 min to fully dissolve.
4. Add 500 µL sterile deionized water to the vial of **Spectinomycin**. Vortex to mix.
5. Measure out 700 mL sterile deionized water with a 1-L measuring cylinder and pour the water into the flask labeled **KIX/SPT**.
6. Add 3 mL sterile deionized water to the vial of **KIX Mix**. Recap and shake to mix.
7. Pour the **KIX Mix** slurry into the **KIX/SPT** labeled flask with the water.
   - **KIX Mix** contains Kanamycin, IPTG, and X-Gal which are hazardous if inhaled.
   - Do not try to pipette the slurry as it will stick to the pipette tip. Pouring the slurry out is more efficient.
8. Repeat **steps 6 - 7** at least two more times to thoroughly rinse the **KIX Mix** vial.
9. Swirl the solution until the insoluble white powder is evenly suspended. Immediately measure out 200 mL using a 1-L measuring cylinder and transfer to the **KIX** labeled flask.
10. Weigh out 7 g **LB agar** powder using a weighing scale.
11. Add the 7 g **LB agar** powder to the **KIX** labeled flask.
12. Add the remaining **LB agar** powder to the **KIX/SPT** labeled flask.
13. Autoclave both flasks on a liquid cycle. Or microwave each flask to boiling three times, taking care not to boil over. The molten liquid should be clear and agar fully dissolved.
14. Leave the two flasks to cool down.
15. While waiting for the flasks to cool, label 8 plates **KIX**, 8 plates **KIX/ARA**, and 40 plates **KIX/SPT**.
16. Once the **KIX** labeled flask has cooled down to around 60˚C, pour the 8 plates labeled **KIX**. Fill the plates to about one-third full (~10 mL).
    - If pouring exactly 10 mL, you may be able to pour up to 10 plates, resulting in 2 extra plates.
17. Add 1 mL rehydrated **Arabinose** to the remaining molten **KIX** LB agar, and swirl to mix.
18. Pour the 8 plates labeled **KIX/ARA**.
    - If pouring exactly 10 mL, you may be able to pour up to 10 plates, resulting in 2 extra plates
19. Add 500 µL rehydrated **Spectinomycin** to the **KIX/SPT** labeled flask once it has cooled down to around 50˚C. Swirl to mix.

20. Pour the 40 plates labeled **KIX/SPT**.
21. Allow the plates to cool down and solidify.
22. Store the plates at room temperature for 1 day to dry. Keep the plates in the dark as the X-Gal is light sensitive.
23. After drying, wrap the plates in aluminum foil to protect from light, and store the plates lid-side down in a 4˚C refrigerator.

Rehydrating the lyophilized *E. coli*

1. Prepare a 100-mL bottle and label it **LB broth**.
2. Measure out 50 mL sterile deionized water with a 50-mL measuring cylinder and pour the water into the bottle labeled **LB broth**.
3. Add the **LB broth** capsule into the water in the bottle and cap the bottle loosely.
4. Autoclave the bottle on a liquid cycle. Or microwave the bottle to boiling three times, taking care not to boil over. The liquid should be clear and the capsule fully dissolved.
5. Leave the bottle to cool to room temperature.
6. Take the lyophilized ***E. coli* HB101-pBRKan** vial out of the freezer.
7. Add 250 µL **LB broth** to the vial of lyophilized *E. coli*.
8. Recap the vial and shake to resuspend the bacteria.
9. Incubate the vial at 37˚C overnight.
10. Store the remainder of the **LB broth** in a 4˚C refrigerator.

## Monday 7/7

### Instructor preparation: Plating out the rehydrated bacteria

Materials:

- 8x LB + Kan + IPTG + X-Gal (**KIX**) agar plates
- 8x LB + Kan + IPTG + X-Gal + Arabinose (**KIX/ARA**) agar plates
- 1x vial of E. coli HB101-pBRKan (rehydrated)
- 2x sterile inoculation loops
- Incubator set at 37˚C

Protocol:

1. Grab the 8 **KIX** labeled plates and the 8 **KIX/ARA** labeled plates, and let them warm to room temperature.
2. Retrieve the *E. coli* vial from the incubator.
3. Using sterile inoculation loops, streak the rehydrated E. coli onto each of the 8 **KIX** and **KIX/ARA** labeled plates.

# Part 3: *In Vivo* Genome Editing of *E. coli* using Cas9 cont.

- Be aware of the huge amount of bacteria that is transferred. Proper streaking technique is required to obtain single colonies.

4. Incubate the plates lid-side down at 37˚C overnight. Be sure the plates are kept in the dark in the incubator.
   - Or at room temperature for 72 hours.

## Tuesday 7/8

### Instructor preparation: Aliquoting reagents for transformation

Materials:

- 1x bottle of **Transformation Solution**
- 1x tube of **pLZDonor**
- 1x tube of **pLZDonorGuide**
- 1x bottle of **LB broth** (prepared above)
- 32x 2-mL microcentrifuge tubes
- Pipettes
- Pipette tips

Protocol:

1. Take out the **Transformation Solution**, **pLZDonor** tube, and **pLZDonorGuide** tube out of the freezer. Take the **LB broth** out of the refrigerator.
2. Thaw out the **Transformation Solution** and store on ice.
3. Allow the **pLZDonor** tube, **pLZDonorGuide** tube, and the **LB broth** warm up to room temperature.
4. For each of the 8 student groups, prepare four 2-mL microcentrifuge tubes and label them **TS**, **LB**, **pD**, **pDG** (32 tubes total).
5. Aliquot 1.2 mL **Transformation Solution** to each of the eight tubes labeled **TS**.
6. Aliquot 1.2 mL sterile **LB broth** to each of the eight tubes labeled **LB**.
7. Aliquot 25 µL **pLZDonor** plasmid to each of the eight tubes labeled **pD**.
8. Aliquot 25 µL **pLZDonorGuide** plasmid to each of the eight tubes labeled **pDG**.
9. Store all solutions at 4˚C until ready for use. Allow the LB broth (**LB**), pLZDonor plasmids (**pD**), and the pLZDonorGuide (**pDG**) to warm up to room temperature before the class. Keep the transformation solution (**TS**) at 4˚C.

Today we will be transforming the *E. coli* with plasmids containing the donor DNA or the donor DNA and sgRNA targeting the lacZ gene. Following transformation, the bacteria will be plated on LB + Kan + IPTG + X-Gal + Spec agar plates to select for successfully transformed bacteria and to observe the blue/white screening.

## Student task: Transformation of the *E. coli*

### Materials

- 1x agar plate labeled **KIX** with bacteria
- 1x agar plate labeled **KIX/ARA** with bacteria
- 1x tube of Transformation solution (**TS**) keep on ice
- 1x tube of pLZDonor plasmid (**pD**)
- 1x tube of pLZDonorGuide plasmid (**pDG**)
- 1x tube of LB broth (**LB**)
- 4x LB + Kan + IPTG + X-Gal + Spec (**KIX/SPT**) agar plates
- 4x 2-mL microcentrifuge tubes
- Pipettes
- Pipette tips
- Ice bucket + ice
- Stopwatch
- Water bath set at 60˚C
- Incubator set at 37˚C

### Protocol:

1. Take four 2-mL microcentrifuge tubes. Label them **A - D** and place them on ice.
2. Add 250 µL ice cold Transformation Solution (**TS** tube) to each tube **A - D**. Return the tubes back on ice.
3. Using a sterile inoculation loop, pick up to five colonies from the **KIX** (Kanamycin, IPTG, X-Gal) plate.
4. Swirl the inoculation loop in tube **A**. Make sure all the bacteria is transferred into the tube, and return the tube back on ice.
5. Repeat **steps 3 - 4**, picking colonies from the **KIX** plate and inoculating tube **B**.
6. Using a sterile inoculation loop, pick up five colonies from **KIX/ARA** (Kanamycin, IPTG, X-Gal, Arabinose) plates.
7. Swirl the inoculation loop in tube **C**. Make sure all the bacteria is transferred into the tube, and return the tube back on ice.
8. Repeat **steps 6 - 7**, picking colonies from the **KIX/ARA** plate and inoculating tube **D**.
9. Add 10 µL pLZDonor plasmid (**pD**) to tube **A**.

# Part 3: *In Vivo* Genome Editing of *E. coli* using Cas9 cont.

10. Close tube **A**, flick the tube three times to mix, and place back on ice.
11. Repeat **steps 9 - 10**, adding pZLDonor plasmid (**pD**) to tube **C**.
12. Add 10 µL pLZDonorGuide plasmid (**pDG**) to tube **B**.
13. Close tube **B**, flick the tube three times to mix, and place back on ice.
14. Repeat **steps 12 - 13**, adding pZLDonorGuide plasmid (**pDG**) to tube **D**.
15. Incubate tubes **A - D** on ice for 10 minutes.
16. Heat shock at 60˚C for 50 sec. Place the tubes into a water bath set at 60˚C.
17. Immediately return the tubes onto ice. Incubate for 2 minutes.
18. Add 250 µL LB broth (**LB**) to tubes **A - D**.
19. Close the tubes, flick the tubes three times to mix, and leave at room temperature from 20 minutes.
20. Take four **KIX/SPT** (Kanamycin, IPTG, X-Gal, Spectinomycin) plates. Label them **A - D**.
    - Remember to label with your initials and date.
21. Gently tap tube **A** to resuspend the bacteria.
22. Transfer 100 µL of liquid from tube **A** onto plate **A**.
23. Using a sterile inoculation loop, spread the liquid evenly across plate **A**.
24. Repeat **steps 21 - 23** with tube **B - D** and plate **B - D**.
25. Turn the plates lid-side down, and incubate at 37˚C overnight.
    - Or at room temperature for 72 hours.

## Wednesday 7/9

Today we will be checking the plates to look for blue and white colonies. To validate that the lacZ gene was edited to have a stop codon, we will use colony PCR and gel electrophoresis to determine the size of the recombined genomic locus.

### Student task: Blue/white screening check

Materials

- 1x agar plate labeled **A** (KIX + pLZDonor)
- 1x agar plate labeled **B** (KIX + pLZDonorGuide)
- 1x agar plate labeled **C** (KIX/ARA + pLZDonor)
- 1x agar plate labeled **D** (KIX/ARA + pLZDonorGuide)

Protocol:

1. Retrieve plates from the 37˚C incubator.
2. Check the plates for color development.
3. If blue and white colonies cannot be distinguished, refrigerate the plates at 4˚C for 1-5 days until a color difference is visible.

# Part 3: *In Vivo* Genome Editing of *E. coli* using Cas9 cont.

## Instructor preparation: Aliquot reagents for PCR

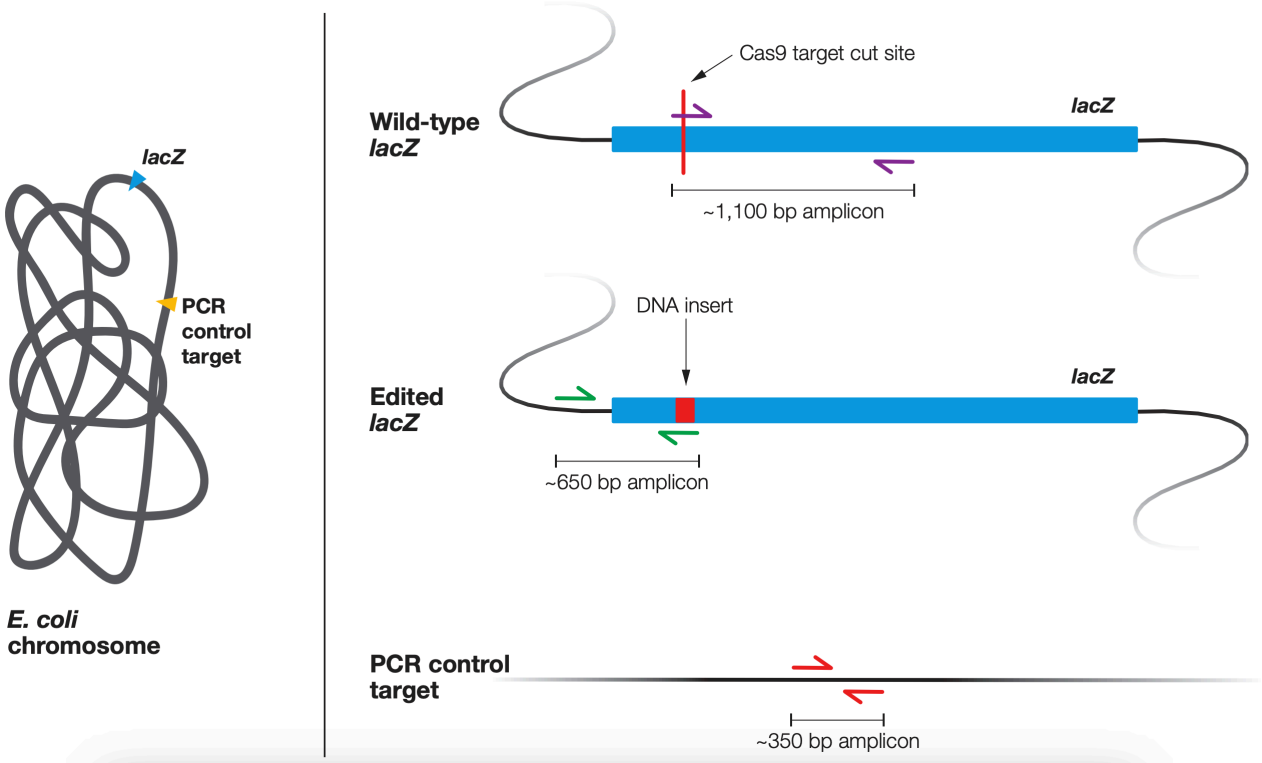
Materials:

- 1x tube of **2x PCR Master Mix**
- 1x tube of **50x Out of the Blue Primer Mix**
- 1x tube of **Positive Control DNA**
- 1x bottle of **InstaGene Matrix**
- **Distilled water**
- 33x 1.5-mL microcentrifuge tube
- Pipettes
- Pipette tips

Protocol:

1. Take the **2x PCR Master Mix**, **50x Out of the Blue Primer Mix**, and **Positive Control DNA** out of the freezer. Take the **InstaGene Matrix** out of the refrigerator.
2. Thaw out the **2x PCR Master Mix**, **50x Out of the Blue Primer Mix**, and **Positive Control DNA**, and store on ice.
3. Take a 1.5-mL microcentrifuge tube and label it **MMP**.
4. Add 735 µL **2x PCR Master Mix** to the tube labeled **MMP**.
5. Add 15 µL **50x Out of the Blue Primer Mix** to the tube labeled **MMP**.
6. Pipet the solution up and down to mix.
7. For each of the 8 student groups, prepare four 1.5-mL microcentrifuge tubes and label them **IG**, **MMP**, **(+)**, **(-)** (32 tubes total).
8. Aliquot 1.3 mL **InstaGene Matrix** to each of the eight tubes labeled **IG**. Transfer 2x 650 µL InstaGene Matrix for the total 1.3 mL.
   - Before pipetting, resuspend the resin by shaking the bottle and stirring manually, or stir on a stir plate using the magnetic stir bar included in the bottle.
9. Aliquot 80 µL **MMP** made in **step 6** to each of the eight tubes labeled **MMP**.
10. Aliquot 15 µL **Positive Control DNA** to each of the eight tubes labeled **(+)**.
11. Aliquot 15 µL **distilled water** to each of the eight tubes labeled **(-)**.

Standard PCR amplifies a specific nucleotide sequence (amplicon) using a single set of primers (usually a pair). Multiplex PCR is the simultaneous amplification of multiple amplicons in a single reaction using a unique primer set for each. As with standard PCR, a primer set targets a particular DNA sequence. Either the absence of a target PCR sequence or the disruption of primer binding sites will prevent amplification of the target PCR sequence. The first primer set detects the unmodified lacZ gene. The second primer detects the modified lacZ gene. And the third set detects an unrelated region as a control for PCR.



# Part 3: *In Vivo* Genome Editing of *E. coli* using Cas9 cont.

## Student task: Genomic DNA extraction and PCR check of edit

Materials:

- 1x agar plate labeled **KIX/ARA** (starter plate)
- 1x agar plate labeled **C** (KIX/ARA + pLZDonor)
- 1x agar plate labeled **D** (KIX/ARA + pLZDonorGuide)
- 1x tube of InstaGene Matrix (**IG**)
- 1x tube of 2x PCR Master Mix and Primer Mix (**MMP**)
- 1x tube of Positive Control DNA **(+)**
- 1x tube of distilled water **(-)**
- 5x 1.5-mL screw-cap tubes
- 7x 0.2-mL PCR tubes
- Vortex mixer
- Microcentrifuge
- Water bath set at 56˚C
- Heat block set at 95˚C
- Thermal cycler
- Pipettes
- Pipette tips

Protocol:

1. Take five 1.5-mL screw-cap tubes and label them **S** (for starter plate), **C**, **D1**, **D2**, **D3**.
2. Flick the InstaGene Matrix (**IG**) tube to evenly resuspend the beads.
3. Add 250 µL InstaGene Matrix (**IG**) to each of the labeled tubes.
    - Flick the tube to resuspend the beads between each pipetting.
4. Use a pipet tip to pick a single colony from the **KIX/ARA** (kanamycin, IPTG, X-Gal, arabinose) plate.
5. Swirl the pipet tip in tube **S**.
6. Repeat **steps 4 - 5**, with plate **C** and add to the tube labeled **C**.
7. Repeat **steps 4 - 5**, with plate **D** and add to the tube labeled **D1**.
8. Repeat **steps 4 - 5**, with plate **D** and add to the tube labeled **D2**.
9. Repeat **steps 4 - 5**, with plate **D** and add to the tube labeled **D3**.
10. Cap the screw-cap tubes.

11. Vortex all tubes for 10 sec to mix.
12. Place the tubes into a water bath set at 56˚C and incubate for 15 minutes.
13. Remove the tubes from the water bath and let them slightly cool.
14. Vortex all tubes for 10 sec to mix.
15. Place the tubes into a water bath set at 95˚C and incubate for 8 minutes.
16. Remove the tubes from the water bath and let them slightly cool.
17. Vortex all tubes for 10 sec to mix.
18. Spin the tubes in a centrifuge at 12,000 xg for 2 minutes.
19. Take seven PCR tubes and label them **S**, **C**, **D1**, **D2**, **D3**, **(+)**, and **(-)**.
20. Add 10 µL PCR master mix with primers (**MMP**) to each of the seven PCR tubes.
21. Take 10 µL of the supernatant in the screw-cap tube **S** and add it to the PCR tube **S**.
    - Do not transfer any of the beads. They will interfere with the PCR.
22. Repeat **step 21** with supernatants of tubes **C**, **D1**, **D2**, and **D3** and add it to the PCR tubes **C**, **D1**, **D2**, and **D3**.
23. Add 10 µL Positive Control DNA **(+)** to the PCR tube labeled **(+)**.
24. Add 10 µL distilled water **(-)** to the PCR tube labeled **(-)**.
25. Cap all PCR tubes.
26. Place the PCR tubes in the thermal cycler and run the following program.

| Step | Temp, Time | |
|---|---|---|
| 1 Initial denature | 98°C, 5 min | 1x |
| 2 Denature | 98°C, 30 s | 35x cycles |
| 3 Anneal | 62°C, 30 s | |
| 4 Extension | 74°C, 1 min | |
| 5 Final extension | 74°C, 5 min | 1x |
| 6 Hold | 10°C, infinity | |

# Part 3: *In Vivo* Genome Editing of *E. coli* using Cas9 cont.

## Instructor preparation: Aliquot reagents for agarose gel

Materials:

- 1x TAE (Often purchasable as liquid concentrate or powder. Follow manufacturer's instructions to prepare 1X TAE Buffer.)
- 10,000x GelRed Nucleic Acid Stain (MilliporeSigma, cat. SCT123)
- 5x Orange G Loading Dye (provided in the kit)
- PCR Molecular Weight Ruler (provided in the kit)
- 24x 1.5-mL microcentrifuge tubes
- Pipettes
- Pipette tips

Protocol:

1. Prepare 1x TAE Buffer
2. Take out the 10,000x GelRed Nucleic Acid Stain.
3. Take the PCR Molecular Weight Ruler and 5x Orange G Loading Dye out of the freezer.
4. Add 50 µL 5x Orange G Loading Dye to the PCR Molecular Weight Ruler tube.
5. Pipet the solution up and down to mix.
6. For each of the 8 student groups, prepare three 1.5-mL microcentrifuge tubes and label them **MWR**, **LD**, and **GR** (24 tubes total).
7. Aliquot 5 µL 10,000x GelRed Nucleic Acid Stain to the tubes labeled **GR**.
8. Aliquot 40 µL 5x Orange G Loading Dye to the tubes labeled **LD**.
9. Aliquot 15 µL PCR Molecular Weight Ruler with the loading dye to the tubes labeled **MWR**.

## Student task: Running agarose gel to analyse the PCR

Materials:

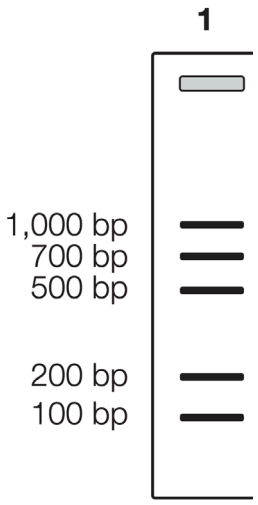
- Agarose - at the weigh station
- 1x TAE - at the front of the class
- 1x tube of 10,000x GelRed Nucleic Acid Stain (**GR**)
- 1x tube of 5x Orange G Loading Dye (**LD**)
- 1x tube of PCR Molecular Weight Ruler (**MWR**)
- Gel electrophoresis chamber
- 1x Gel tray
- 1x 12-well comb
- 1x 150-mL conical flask
- 1x 50-mL measuring cylinder
- Microwave
- Power pack
- Plastic wrap
- Pipettes
- Pipette tips

Protocol:

1. While the thermal cycler program is running, pour a 1% agarose gel.
2. Prepare a gel mold with a 12-well comb
3. Weigh out 0.5 g agarose into a flask.
4. Measure out 50 mL 1x TAE buffer in a measuring cylinder and add it to the flask with agarose.
5. Cover the top of the flask with plastic wrap and microwave until it just starts to boil. Do not let it boil over.
6. Repeat **step 5** until the agarose is fully dissolved,
7. Allow the flask to cool down to about 60˚C.
8. Add 5 µL GelRed stain (**GR**) to the cooled flask and swirl to mix.
9. Pour the mixture into the gel mold.
10. Leave the gel to cool and solidify.
11. Once the PCR has finished, add 5 µL 5x Orange G Loading Dye (**LD**) to each of the seven PCR tubes.
12. Pipet gently to mix.
13. Place the 1% TAE agarose gel into the electrophoresis tank and fill the tank with 1x TAE buffer.

# Part 3: *In Vivo* Genome Editing of *E. coli* using Cas9 cont.

14. Using a new pipet tip for each sample, load 15 µL of each of the following samples
    1 Molecular Weight Ruler (**MWR**)
    2 Positive Control DNA **(+)**
    3 PCR Sample (**S**)
    4 PCR Sample (**C**)
    5 PCR Sample (**D1**)
    6 PCR Sample (**D2**)
    7 PCR Sample (**D3**)
    8 Negative control **(-)**
15. Place the lid on the electrophoresis tank.
16. Run the gel at 120V for 20 minutes.
17. Visualize the gel under UV light/blue light.
18. Examine your gel and celebrate! You're done!




Use this as a reference for how the PCR Molecular Weight Ruler  runs on a 1% agarose gel.

# Notes

# Part 4: Applications of CRISPR in plant research

We now move to CRISPR application in plant research. And we will focus on how we can design guide RNAs and clone them for use in Arabidopsis.

## Tuesday 7/8

### Student task: Designing a guide to target AT4G14210 in Arabidopsis thaliana

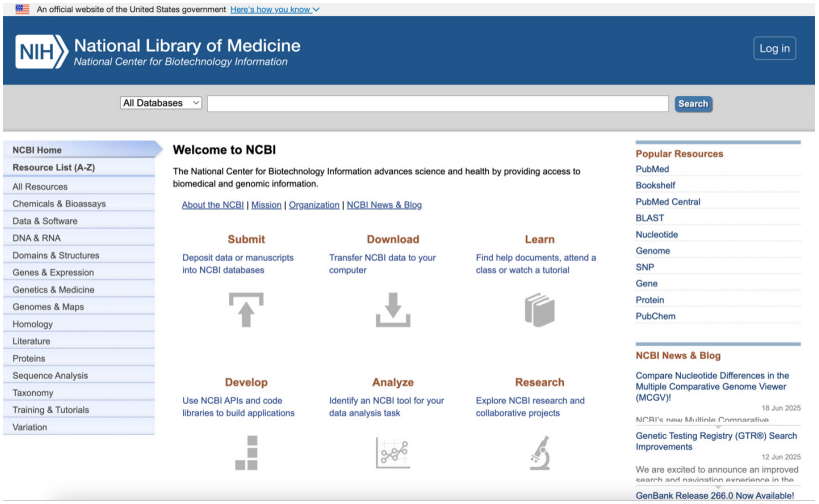
Materials:

- Laptop

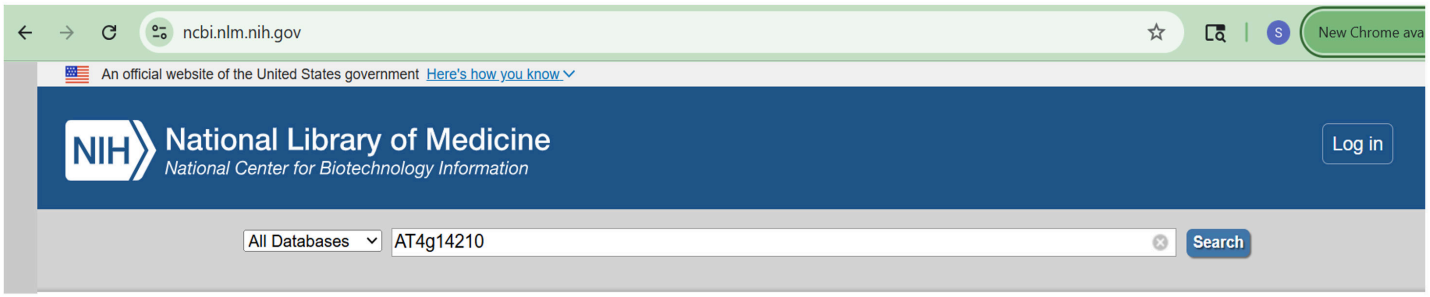
Protocol:

Step 1: Search for the Gene on NCBI

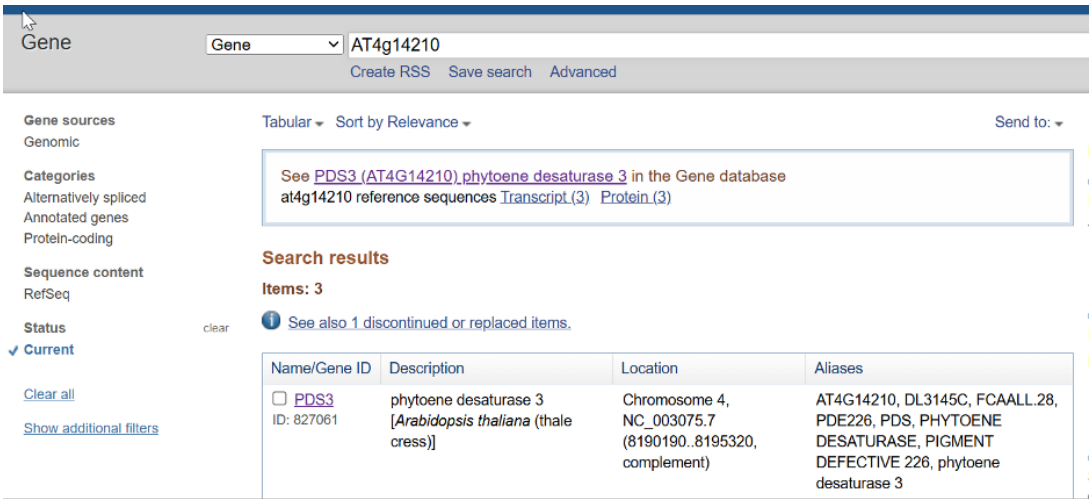
1. Go to the NCBI website ***https://www.ncbi.nlm.nih.gov/***



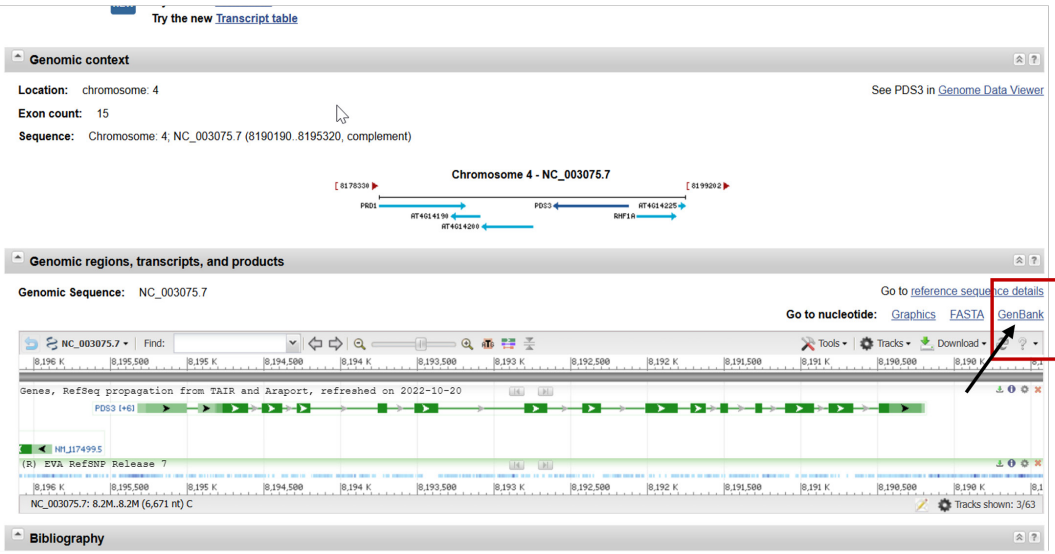

2. In the **search bar**, enter the name of your gene of interest (e.g., Arabidopsis PHYTOENE DESATURASE PDS3 or AT4g14210) and press Enter. Select "**Gene**" from the dropdown menu (instead of "All Databases") for more targeted results.



3. From the results list, select the appropriate gene entry (i.e **AT4G14210 – Arabidopsis thaliana** or ID 827061).

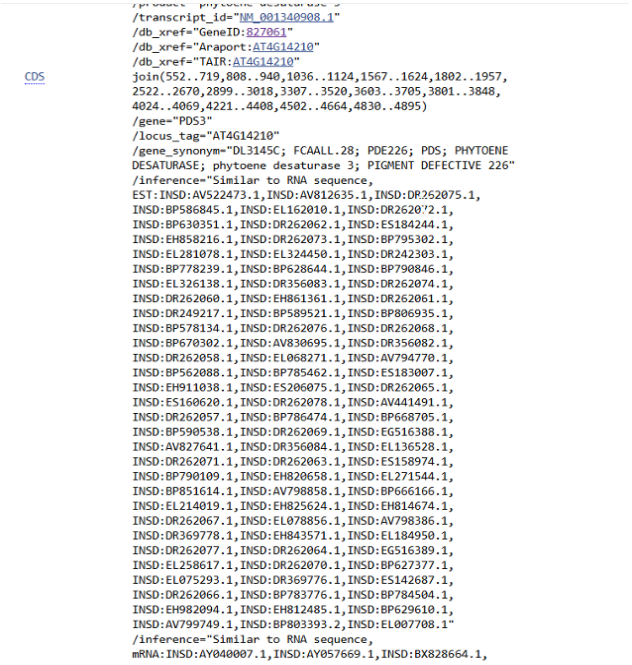



4. From the list click on "gene" to get more information about the gene. On the gene record page, locate and click the link to the **GenBank record** (found under "Genomic regions, transcripts, and products").



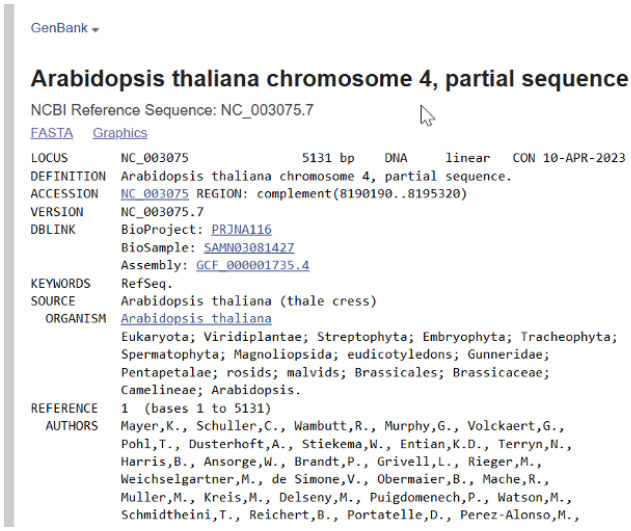
# Part 4: Applications of CRISPR in plant research cont.

5. Scroll down to the **CDS (coding sequence)** feature in the GenBank view and record the **start codon position (e.g., 552, ATG)** and **stop codon position (e.g., 4895)**. You will use this information to annotate the start and stop sites of the gene in Benchling.



```
                     /transcript_id="NM_001340908.1"
                     /db_xref="GeneID:827061"
                     /db_xref="Araport:AT4G14210"
                     /db_xref="TAIR:AT4G14210"
CDS                  join(552..719,808..940,1036..1124,1567..1624,1802..1957,
                     2522..2670,2899..3018,3307..3520,3603..3705,3801..3848,
                     4024..4069,4221..4408,4502..4664,4830..4895)
                     /gene="PDS3"
                     /locus_tag="AT4G14210"
                     /gene_synonym="DL3145C; FCAALL.28; PDE226; PDS; PHYTOENE
                     DESATURASE; phytoene desaturase 3; PIGMENT DEFECTIVE 226"
                     /inference="Similar to RNA sequence,
                     EST:INSD:AV522473.1,INSD:AV812635.1,INSD:DR262075.1,
                     INSD:BP586845.1,INSD:EL162010.1,INSD:DR262072.1,
                     INSD:BP630351.1,INSD:DR262062.1,INSD:ES184244.1,
                     INSD:EH858216.1,INSD:DR262073.1,INSD:BP795302.1,
                     INSD:EL281078.1,INSD:EL324450.1,INSD:DR242303.1,
                     INSD:BP778239.1,INSD:BP628644.1,INSD:BP790846.1,
                     INSD:EL326138.1,INSD:DR356083.1,INSD:DR262074.1,
                     INSD:DR262060.1,INSD:EH861361.1,INSD:DR262061.1,
                     INSD:DR249217.1,INSD:BP589521.1,INSD:BP806935.1,
                     INSD:BP578134.1,INSD:DR262076.1,INSD:DR262068.1,
                     INSD:BP670302.1,INSD:AV830695.1,INSD:DR356082.1,
                     INSD:DR262058.1,INSD:EL068271.1,INSD:AV794770.1,
                     INSD:BP562088.1,INSD:BP785462.1,INSD:ES183007.1,
                     INSD:EH911038.1,INSD:ES206075.1,INSD:DR262065.1,
                     INSD:ES160620.1,INSD:DR262078.1,INSD:AV441491.1,
                     INSD:DR262057.1,INSD:BP786474.1,INSD:BP668705.1,
                     INSD:BP590538.1,INSD:DR262069.1,INSD:EG516388.1,
                     INSD:AV827641.1,INSD:DR356084.1,INSD:EL136528.1,
                     INSD:DR262071.1,INSD:DR262063.1,INSD:ES158974.1,
                     INSD:BP790109.1,INSD:EH820658.1,INSD:EL271544.1,
                     INSD:BP851614.1,INSD:AV798858.1,INSD:BP666166.1,
                     INSD:EL214019.1,INSD:EH825624.1,INSD:EH814674.1,
                     INSD:DR262067.1,INSD:EL078856.1,INSD:AV798386.1,
                     INSD:DR369778.1,INSD:EH843571.1,INSD:EL184950.1,
                     INSD:DR262077.1,INSD:DR262064.1,INSD:EG516389.1,
                     INSD:EL258617.1,INSD:DR262070.1,INSD:BP627377.1,
                     INSD:EL075293.1,INSD:DR369776.1,INSD:ES142687.1,
                     INSD:DR262066.1,INSD:BP783776.1,INSD:BP784504.1,
                     INSD:EH982094.1,INSD:EH812485.1,INSD:BP629610.1,
                     INSD:AV799749.1,INSD:BP803393.2,INSD:EL007708.1"
                     /inference="Similar to RNA sequence,
                     mRNA:INSD:AY040007.1,INSD:AY057669.1,INSD:BX828664.1,
```

6. Next, click on the "**FASTA**" link at the top of the GenBank record to view the nucleotide sequence. **Copy the full nucleotide sequence** and paste it into a Word document or plain text editor for future use in Benchling.



GenBank

**Arabidopsis thaliana chromosome 4, partial sequence**

NCBI Reference Sequence: NC_003075.7

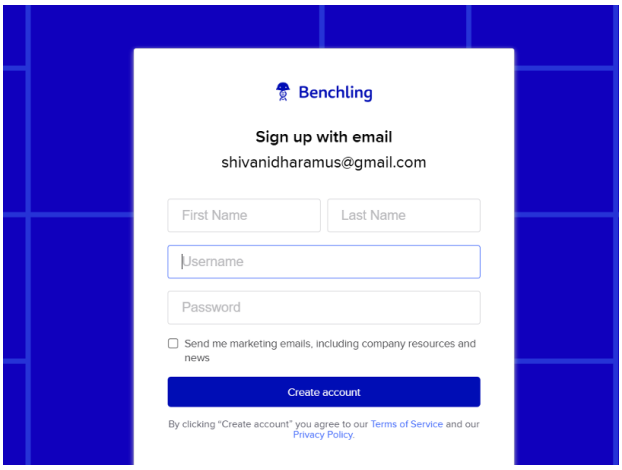
FASTA Graphics

```
LOCUS       NC_003075              5131 bp    DNA     linear   CON 10-APR-2023
DEFINITION  Arabidopsis thaliana chromosome 4, partial sequence.
ACCESSION   NC_003075 REGION: complement(8190190..8195320)
VERSION     NC_003075.7
DBLINK      BioProject: PRJNA116
            BioSample: SAMN03081427
            Assembly: GCF_000001735.4
KEYWORDS    RefSeq.
SOURCE      Arabidopsis thaliana (thale cress)
  ORGANISM  Arabidopsis thaliana
            Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
            Spermatophyta; Magnoliopsida; eudicotyledons; Gunneridae;
            Pentapetalae; rosids; malvids; Brassicales; Brassicaceae;
            Camelineae; Arabidopsis.
REFERENCE   1  (bases 1 to 5131)
  AUTHORS   Mayer,K., Schuller,C., Wambutt,R., Murphy,G., Volckaert,G.,
            Pohl,T., Dusterhoft,A., Stiekema,W., Entian,K.D., Terryn,N.,
            Harris,B., Ansorge,W., Brandt,P., Grivell,L., Rieger,M.,
            Weichselgartner,M., de Simone,V., Obermaier,B., Mache,R.,
            Muller,M., Kreis,M., Delseny,M., Puigdomenech,P., Watson,M.,
            Schmidtheini,T., Reichert,B., Portatelle,D., Perez-Alonso,M.,
```

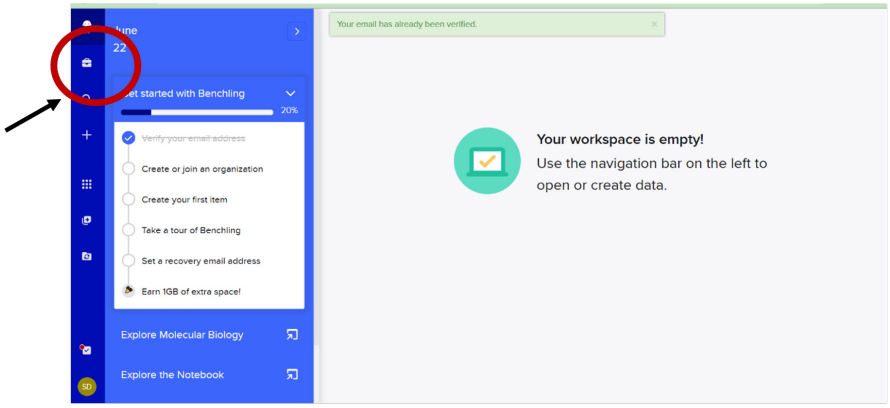
**Note*** - There are several free Electronic Lab Notebooks (ELN) on the market, we use Benchling as an example here because it is free and has molecular biology tools. Here is a list of other ELNs from the Harvard Medical School. https://zenodo.org/records/4723753 Step 2: Set Up a Benchling Account and Project

## Step 2: Set Up a Benchling Account and Project

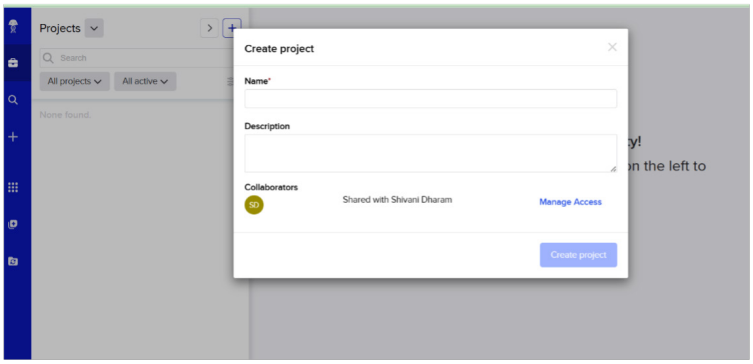
1. Go to benchling ( *https://www.benchling.com*). Sign up for a **free academic account** using your institutional email address. Verify your email to access the Benchling workspace. You can also create your Lab group and ask students to join it so you can view their work.




2. In your workspace, click on the "**Projects**" tab and then click the **"+" icon** to create a new project.



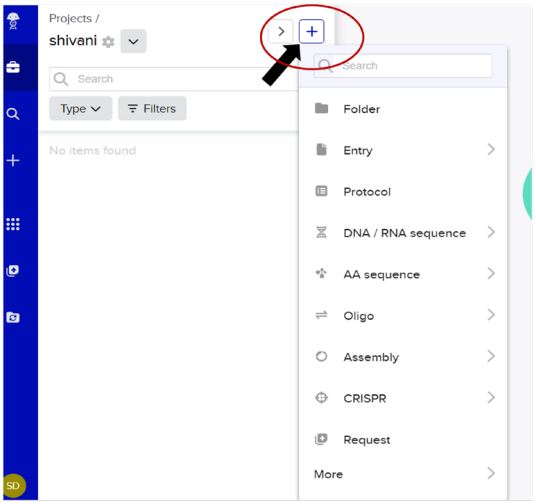

3. Enter a **project name** (e.g., "PDS3 Project"), and provide a short description. You may also set **sharing permissions** for collaboration.



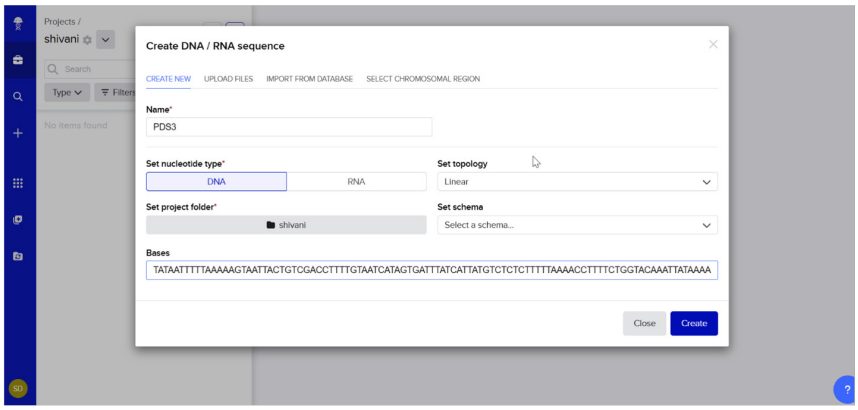
# Part 4: Applications of CRISPR in plant research cont.

Step 3: Import and Annotate the Gene in Benchling

1. Inside your project, click **“+”** and select **“DNA Sequence”**.



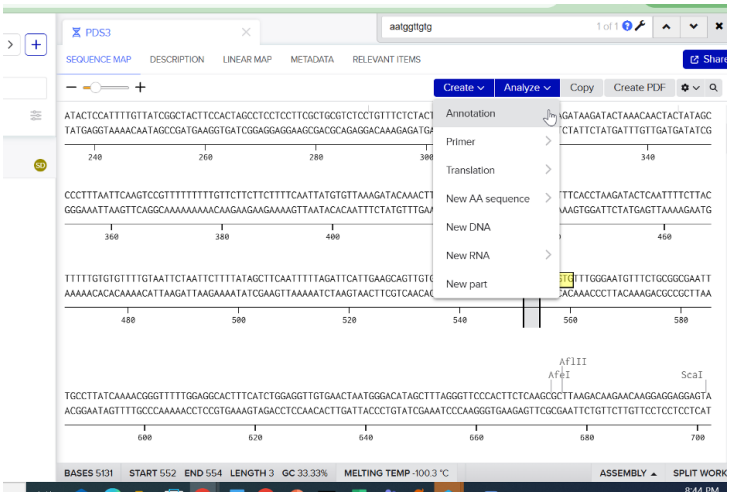

2. Here, you can either upload the sequence file or copy and paste the nucleotide sequence directly. Paste the nucleotide sequence you copied from NCBI. Name the sequence (e.g., “PDS3 DNA”), set molecule type to **DNA**, and choose the topology as **Linear**.



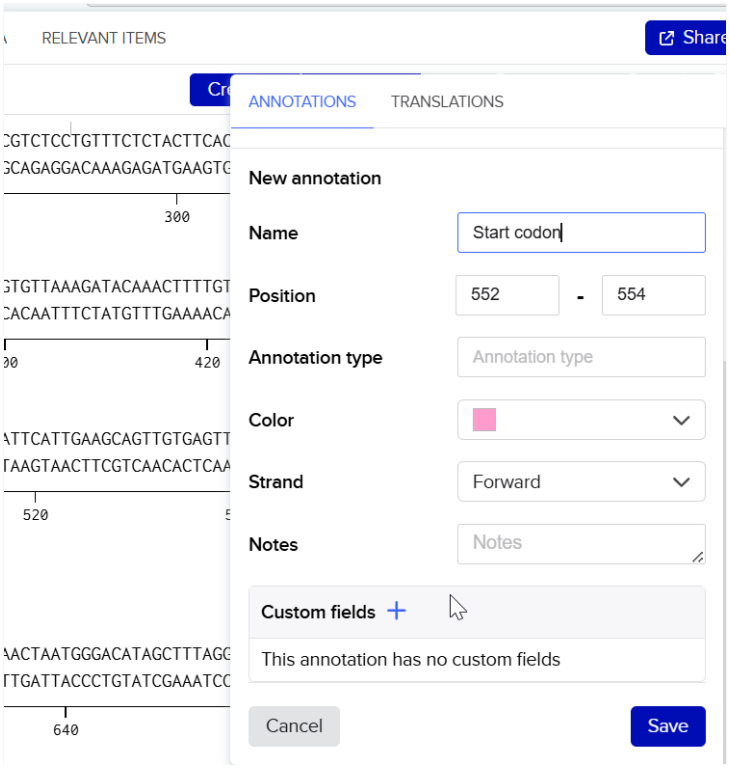

Tip: Use the NCBI GenBank record to **identify a few bases near the CDS start site** (e.g., aatggttgtg near ATG) to help you locate the start codon.

```
                     "
ORIGIN
        1 ctttggtggg caaaaacata ttagctgaga ggtcaatttc tttttcccct aaaccaaatt
       61 acgttgagat gcatggtctc tctctactca attaaccaaa taaggaaaag aatcatatgg
      121 tcatcaattc gtaaatcaaa attttaattt gtgtggtatt taatccatct acatgtttcg
      181 taagcaacaa aagagcttgg tctgaaaacc aaacaagacc atatgggcac tcgaatactc
      241 cattttgtta tcggctactt ccactagcct cctccttcgc tgcgtctcct gtttctctac
      301 ttcacgatta ctcgctaggt aaaagataag atactaaaca actactatag ccccttttaat
      361 tcaagtccgt ttttttttgt tcttcttctt ttcaattatg tgttaaagat acaaacttttt
      421 gtctgatttg cttccaccgg tttcacctaa gatactcaat tttcttactt tttgtgtgtt
      481 ttgtaattct aattcttttta tagcttcaat ttttagattc attgaagcag ttgtgagtta
      541 agttggagaa aatggttgtg tttgggaatg tttctgcggc gaatttgcct tatcaaaacg
```

3. Use Benchling's search function to find the **ATG start codon**, select it, and click **"Create" > "Create Annotation"**. Name the annotation **"Start Codon"**.




Then annotate as start codon and save.

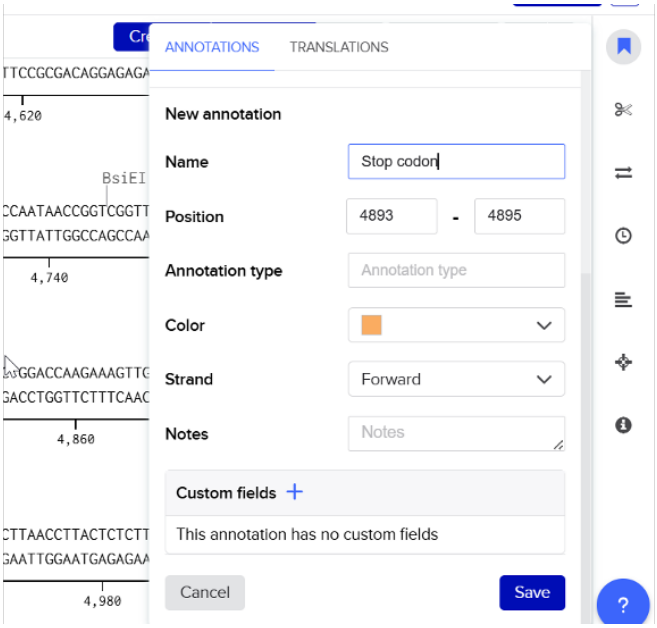


# Part 4: Applications of CRISPR in plant research cont.

4. Similarly, find the **stop codon** near the end of the coding sequence (e.g., tag, taa, or tga), and annotate it as **"Stop Codon"**. Copy few bases as shown in the figure near stop codon

```
4561 ttgaaggatt ctacttagct ggagattaca caaaacagaa gtacttagct tccatggaag
4621 gcgctgtcct ctctggcaaa ttctgctctc agtctattgt tcaggtaaac acaagcttgc
4681 cattcaaata gttaaccagt aaccttgttc tctgtttctt gttggaatat atcgccaata
4741 accggtcggt ttacctgatt ttgtctcact attatactgt tccggttatg tagagaaatt
4801 gctaaagtca gaatttactg gttatacagg attacgagct actggctgcg tctggaccaa
4861 gaaagttgtc ggaggcaaca gtatcatcat catgagaaga ggacaaaact taaagatgat
4921 ttgcttgtaa gcattattat ttgtgtataa atctcattgc aatccaaact taaccttact
4981 ctcttcagta aatgaatctc acagatttga catctcacgt ttctgtcaat tttataattt
5041 ttaaaaagta attactgtcg acctttttgta atcatagtga tttatcatta tgtctctctt
5101 tttaaaacct tttctggtac aaattataaa a
```

Annotate the Stop codon as shown in the figure below.

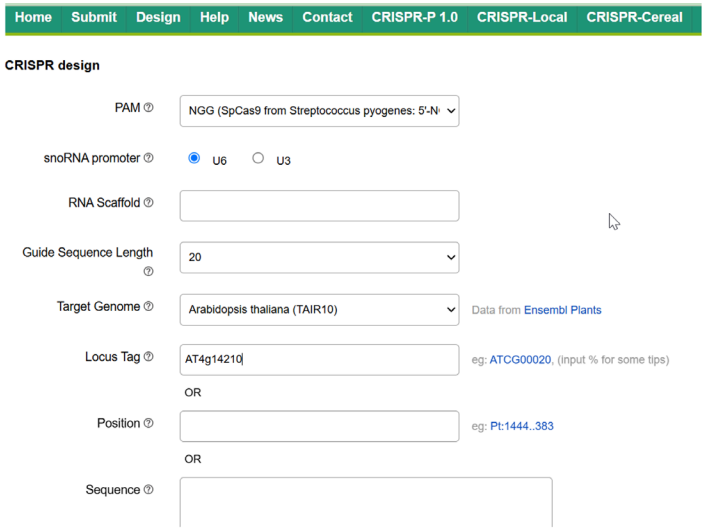


## Step 4: Design Guide RNAs Using CRISPR-P 2.0

The session is focused on the selection of guide RNA (gRNA) and primer design to target the AtPDS3 gene. The course used the pDIRECT_23C vector with the Streptococcus pyogenes SpCas9 enzyme, requiring the presence of an 'NGG' protospacer adjacent motif (PAM) site. This introduces online gRNA design tools, such as CRISPR-P (http://crispr.hzau.edu.cn/cgi-bin/CRISPR2/CRISPR) and will help guide through the process of designing an efficient gRNA. Alternate websites include CRISP-OR.

1. Go to the CRISPR-P 2.0 tool. http://crispr.hzau.edu.cn/CRISPR2/

2. Select the **"CRISPR-P 2.0"** tool for guide RNA design.




3. Choose your options as follows:
   - **PAM sequence:** NGG
   - **Promoter:** U6
   - **Guide length:** 20 nt
   - **Genome:** Select your species (e.g., Arabidopsis thaliana or Solanum lycopersicum)

### Note* Ideal Characteristics of Guide RNAs (gRNAs)

When designing guide RNAs for CRISPR-Cas9 genome editing, consider the following criteria to ensure high efficiency and specificity:

- **Target Location:** Select gRNAs that target **exonic regions near the 5' end** of the gene. This increases the likelihood of producing a loss-of-function mutation by disrupting translation early in the coding sequence.
- **PAM Requirement:** For **SpCas9**, the protospacer adjacent motif (**PAM**) must be **NGG**. The gRNA sequence must lie **immediately upstream** (5') of the NGG PAM to be recognized and cleaved by the Cas9 protein.
- **GC Content:** Optimal gRNAs have a **GC content between 30–70%**. This range ensures adequate thermodynamic stability without introducing strong secondary structures like hairpins that may reduce targeting efficiency.
- **Off-Target Specificity:** Ideal gRNAs have **no significant off-target matches** in the genome. If off-target sites are present:
  - They should have **at least 4–5 mismatches** compared to the guide sequence.
  - Mismatches are most critical within the **seed region** (the first 5 nucleotides closest to the PAM).
  - **Avoid gRNAs** with predicted off-target binding sites located **in coding regions** of other genes, as this may lead to unintended gene disruption.

# Part 4: Applications of CRISPR in plant research cont.

4. Enter the **gene name, locus ID, or paste the nucleotide sequence** and click **“Submit.”**

5. The tool will return a list of guide RNAs with details including:
   - Guide RNA sequence
   - On-target efficiency
   - GC content
   - Number of predicted off-targets
   - Mismatch positions
   - Genomic context (exonic/intronic/intergenic)

6. Choose guides with:
   - **High on-target efficiency**
   - **Low off-target potential**
   - If possible, guides located in **intronic or non-essential exonic regions**

## Thursday 7/10

To clone a guide RNA targeting AtPDS3 using the Golden gate system

### Instructor preparation: Aliquoting PCR reagents

Materials:

- pDIRECT_23C plasmid DNA template
- Primers at 110 µM
  - **Primer 1F (oCmYLCV):** TGCTCTTCGCGCTGGCAGACATACTGTCCCAC
  - **Primer 2R (CSY_guide6):** TGGTCTCCCAATCCAGCCAACTGCCTATACGGCAGTGAAC
  - **Primer 3F (Rep_Guide6):** TGGTCTCAATTGTCAACTGCGTTTTAGAGCTAGAAATAGC
  - **Primer 4R (CSY_Term):** TGCTCTTCTGACCTGCCTATACGGCAGTGAAC
- 2x Phusion High-Fidelity PCR Master Mix (NEB, cat. M0531S)
- Distilled water
- 70x 1.5-mL microcentrifuge tubes
- Pipettes
- Pipette tips

Protocols:

1. Take the 2x Phusion High-Fidelity PCR Master Mix, pDIRECT_23 plasmid, Primer 1F, Primer 2R, Primer 3F, and Primer 4R out of the freezer.
2. For each of the 10 student groups, prepare seven 1.5-mL microcentrifuge tubes and label them **H2O**, **MM**, **pD23**, **P1F**, **P2R**, **P3F**, and **P4R** (70 tubes total).
3. Aliquot 50 µL distilled water to the tubes labeled **H2O**.
4. Aliquot 60 µL 2x Phusion High-Fidelity PCR Master Mix to the tubes labeled **MM**.
5. Aliquot 5 µL 110 µM pDIRECT_23 plasmid to the tubes labeled **pD23**.
6. Aliquot 5 µL 110 µM Primer 1F to the tubes labeled **P1F**.
7. Aliquot 5 µL 110 µM Primer 2R to the tubes labeled **P2R**.
8. Aliquot 5 µL 110 µM Primer 3F to the tubes labeled **P3F**.
9. Aliquot 5 µL 110 µM Primer 4R to the tubes labeled **P4R**.

# Part 4: Applications of CRISPR in plant research cont.

To amplify gRNAs targeting the PDS3 gene using the pDIRECT_23C plasmid as a template, with specific primers incorporating the Cauliflower mosaic yellow leaf curl virus (CmYLCV) promoter and the 35S terminator, for downstream cloning and transformation.

## Student task: Perform PCR to amplify gRNAs

Materials:

- 1x tube of pDIRECT_23C plasmid DNA
- 1x tube of 110 µM **Primer 1F**
- 1x tube of 110 µM **Primer 2R**
- 1x tube of 110 µM **Primer 3F**
- 1x tube of 110 µM **Primer 4R**
  - **Primer 1F (oCmYLCV):** TGCTCTTCGCGCTGGCAGACATACTGTCCCAC
  - **Primer 2R (CSY_guide6):** TGGTCTCCCAATCCAGCCAACTGCCTATACGGCAGTGAAC
  - **Primer 3F (Rep_Guide6):** TGGTCTCAATTGTCAACTGCGTTTTAGAGCTAGAAATAGC
  - **Primer 4R (CSY_Term):** TGCTCTTCTGACCTGCCTATACGGCAGTGAAC
- 1x tube of distilled water
- 2x 0.2-mL PCR tubes
- Pipettes
- Pipette tips
- Ice bucket + ice
- Thermal cycler

Protocol:

1. Label two PCR tubes clearly with identification labels.
2. Follow the table below and add reagents to the tubes.

| Component | Reaction 1 (µL) | Reaction 2 (µL) |
|---|---|---|
| Water | 18 | 18 |
| Primer 1F | 2.5 | - |
| Primer 2R | 2.5 | - |
| Primer 3F | - | 2.5 |
| Primer 4R | - | 2.5 |
| pDIRECT_23C plasmid template | 2 | 2 |
| 2X Phusion Polymerase Mix | 25 | 25 |
| Total Volume | 50 | 50 |

3. Use a P20 micropipette to add **18 µL** of water into each PCR tube.
4. Add **2.5 µL of Primer 1F** and **2.5 µL of Primer 2R** into **Tube 1**.
   Add **2.5 µL of Primer 3F** and **2.5 µL of Primer 4R** into **Tube 2**.
5. Add **2 µL of pDIRECT_23C plasmid DNA template** to both PCR tubes.
6. Add **25 µL of 2X Phusion Polymerase Mix** to each tube to reach a final reaction volume of 50 µL.
7. Always use a new pipette tip for each component to prevent cross-contamination.
8. Briefly spin the tubes using a **mini centrifuge** for approximately **10 seconds** to mix components.
9. Load the PCR tubes into the **thermal cycler** and run the following program.

| Step | Temp, Time | |
|---|---|---|
| 1 Initial denature | 98 °C, 1 min | 1x |
| 2 Denature | 98 °C, 10 s | 35x cycles |
| 3 Anneal | 60 °C, 15 s | |
| 4 Extension | 72 °C, 15 s | |
| 5 Final extension | 72°C, 2 min | 1x |
| 6 Hold | 4°C, infinity | |

10. Start the PCR run and allow it to complete as per the programmed cycle.
11. Later run it on the gel to see the amplification

# Part 4: Applications of CRISPR in plant research cont.

## Golden Gate Assembly

Golden Gate cloning is a molecular technique that utilizes Type IIS restriction enzymes to assemble multiple DNA fragments into a desired vector in a single, directional reaction. BsaI and SapI are examples of Type IIS enzymes, recognizing the sequences 5'-GGTCTC-3' and 5'-GCTCTTC-3', respectively. These enzymes cut outside of their recognition sites, generating unique sticky ends that facilitate seamless and efficient fragment assembly.

### Instructor preparation: Aliquoting Golden Gate assembly reagents

Materials:

pDIRECT_23C plasmid DNA template

2x T7 Ligase Buffer

Distilled water

30x 1.5-mL microcentrifuge tubes

Pipettes

Pipette tips

Protocols:

1. Take the pDIRECT_23 plasmid and 2x T7 Ligase Buffer out of the freezer.
2. For each of the 10 student groups, prepare three 1.5-mL microcentrifuge tubes and label them **H2O**, **pD23**, and **T7LB** (30 tubes total).
3. Aliquot 30 µL distilled water to the tubes labeled **H2O**.
4. Aliquot 5 µL 100 ng/µL pDIRECT_23 plasmid to the tubes labeled **pD23**.
5. Aliquot 15 µL 2x T7 Ligase Buffer to the tubes labeled **T7LB**.

### Student task: Golden gate assembly

Materials:

- PCR products diluted 10-fold
  - **add 1 uL** of each PCR product and 9 uL of Nuclease free water. (Here we have two PCR products from previous reaction)
- 1x tube of pDIRECT_23C plasmid DNA
- 1x tube of T7 Ligase Buffer
- BsaI - at the front of the classroom
- SapI - at the front of the classroom

- T7 Ligase - at the front of the classroom
- 3x 0.2-mL PCR tubes
- Distilled water
- Pipettes
- Pipette tips
- Ice bucket + ice
- Thermal cycler

Protocol:

1. Dilute each PCR product from previous step by 10-fold by adding 1 µL of PCR product to 9 µL of nuclease-free water and label the tubes properly.
2. Label one PCR tube for setting up the entire reaction.

| Component | Reaction (µL) |
|---|---|
| Water | 5 |
| PCR product 1 (diluted 10 times) | 0.5 |
| PCR product 2 (diluted 10 times) | 0.5 |
| BsaI | 0.5 |
| SapI | 0.5 |
| Template (100ng) | 2 |
| T7 DNA Ligase | 1 |
| 2x T7 DNA Ligase Buffer | 10 |
| Total Volume | 20ß |

3. Add 5 µL of nuclease-free water and 10 µL of 2x T7 DNA ligase buffer to the labeled tube.
4. Add 0.5 µL of each diluted PCR product and 2 µL (100ng) of the pDIRECT23C vector, ensuring all components are well mixed.
5. Add 0.5 µL of SapI, 0.5 µL of BsaI, and 1 µL of T7 DNA ligase, at the front of the class.
   - **Note:** SapI enzyme has to be mixed by pipetting up and down several times before adding to the reaction.
6. Close the PCR tube and mix the reaction by pipetting up and down several times. Briefly spin it down to collect all contents at the bottom.
7. Place the PCR tube in the thermocycler and run the following program:

| | | |
|---|---|---|
| Step 1-2, 10x cycles | 37 °C | 5 minutes |
| | 25 °C | 10 minutes |
| | 4 °C | Hold |

# Part 4: Applications of CRISPR in plant research cont.

## Transforming *E. coli* TOP10 competent cells using heat-shock method.

### Instructor preparation: Aliquoting transformation reagents

Materials:

- SOC Media
- 10x 1.5-mL microcentrifuge tubes
- Pipettes
- Pipette tips

Protocols:

1. For each of the 10 student groups, prepare one 1.5-mL microcentrifuge tube and label them **SOC** (10 tubes total).
2. Aliquot 500 µL SOC Media to the tubes labeled **SOC**.

### Student task: transformation

Materials:

- Tube of Golden Gate cloning product (from above)
- 1x tube of OneShot TOP10 Competent cells
- 1x tube of SOC Media
- 1x LB + Kan agar plate
- Water bath set at 42˚C
- Shaking incubator set at 37˚C
- Incubator set at 37˚C
- Ice bucket + ice
- Spreader
- Pipettes
- Pipette tips

Protocol:

Before starting the procedure.

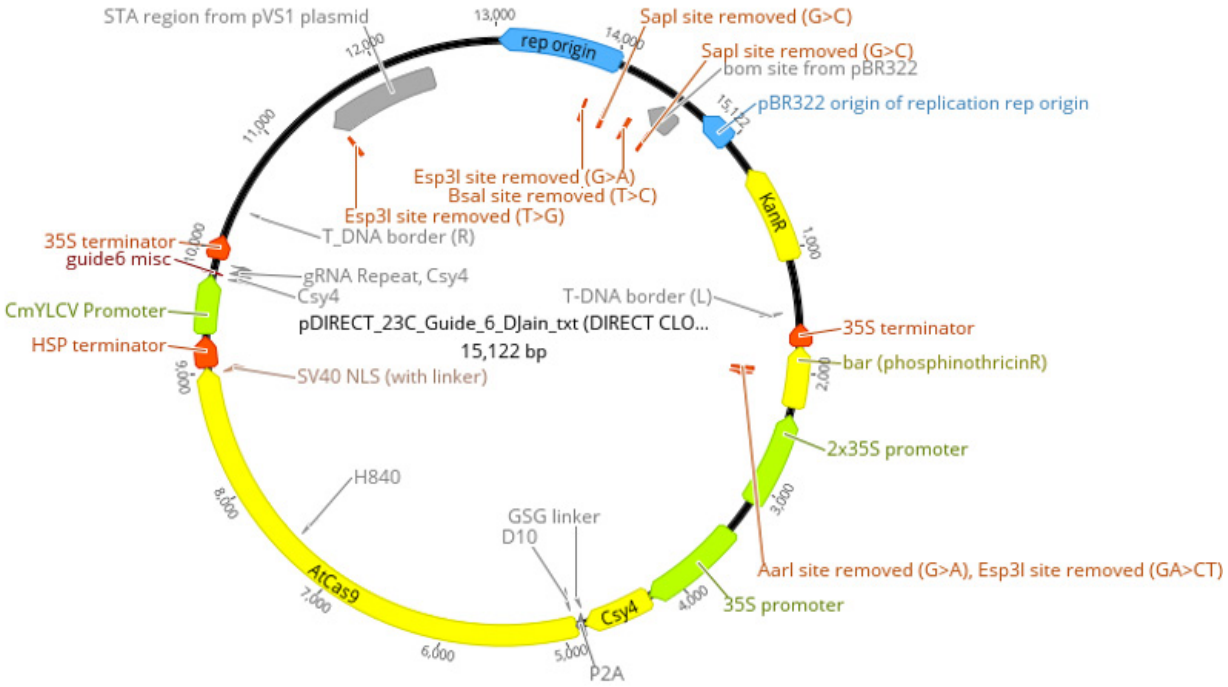
1. Equilibrate a hot plate bath to 42°C.
2. Warm the vial of SOC Medium.
3. Warm the selective plates in a 37°C incubator for 30 minutes (use 1 or 2 plates for each transformation).

Transform competent cells

1. Thaw one vial of One Shot™ TOP10 chemically competent cells for one transformation on ice.
2. Add 5 µL of the golden gate cloning product into (10 pg to 100 ng) a vial of One Shot™ cells and mix gently by tapping. Do not mix by pipetting.
3. Incubate the vial(s) on ice for 15 minutes.
4. Heat-shock the cells for 45 seconds at 42°C without shaking.
5. Remove the vial(s) from the 42°C bath and place them on ice for 2 minutes.
6. Add 250 µL of pre-warmed S.O.C. Medium to each vial.
7. Cap the vial(s) tightly and shake horizontally at 37°C for 30 minutes at 250 rpm in a
8. shaking incubator. Centrifuge the tube at 4000 RPM for 6-8 minutes.
9. Decant the supernatant and add 100 µL of SOC to the tube. And resuspend the pellet completely to dissolve in SOC media. Spread the entire bacterial pellet on a pre-warmed LB media plate containing kanamycin antibiotic and incubate overnight at 37°C without shaking.
10. Invert the selective plate(s) and incubate at 37°C overnight.

# Part 4: Applications of CRISPR in plant research cont.

Vector map of pDIRECT23C vector with guide 6 targeting PDS3 gene.




Gel image showing the amplification of guide RNAs targeting PDS3 gene along with Cm YLC promotor and 35 s terminator.

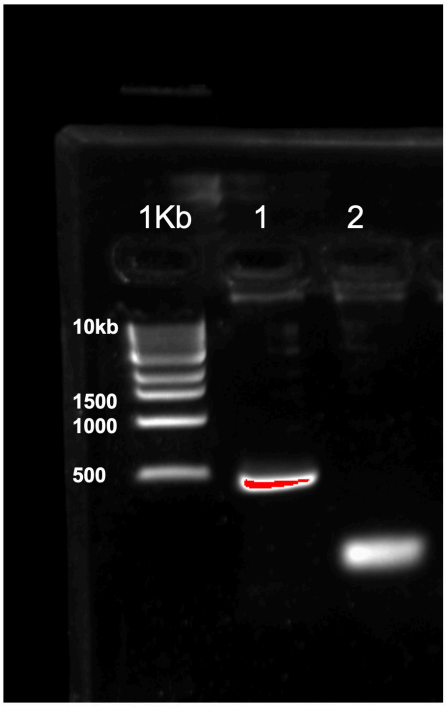
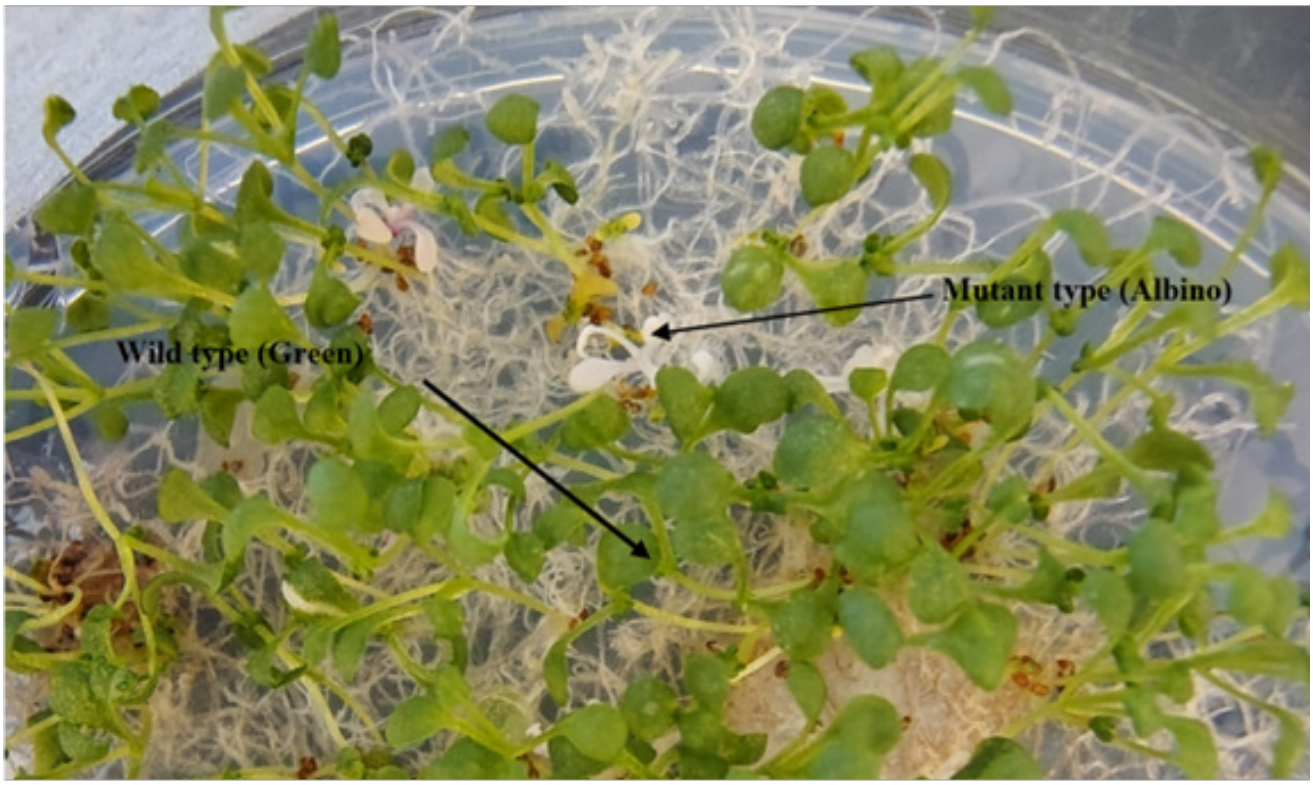


Image showing Arabidopsis wild-type and albino mutant plants with mutations in the PDS3 gene.



# Supplementary Teaching Materials

## Report Details:

Provided is an example of an outline for a report to be written up by the students.

Complete a report outlining this week's experimental results. Below is an outline of the report format. Please be brief and write succinctly, the answer to each question should be no more than 250 words.

Introduce the topic of this week's experiment. What is being tested and how does this relate back to real-world questions or the lectures?

Include figures summarizing your experimental results and describe the outcome of each experiment. Be sure to label all figures (title, lanes, bands, etc.).

If an experiment did not conclude as you expected, explain what may have impacted the outcome and what could be done differently next time.

Suggest alternative controls to the ones included.

Conclude your report by suggesting an additional experiment. Many experiments can raise more questions than they answer, so what new question can you test and how would you test that question?

# Exercise Questions

## Part 1: CRISPR Immunity

1. Why are IPTG and arabinose added to induce the expression?
2. What would happen if we sequenced your PCR products from the liquid culture?
3. What does CRISPR stand for, and how does it relate to the pattern we see on the gel?
4. What is going on at the sequencing facility? How does Sanger sequencing work?
5. Why do we only use one primer in the sequencing reaction?
6. Why do you need to do a PCR cleanup before you sequence?
7. If any of your PCRs didn't work, what do you think went wrong? Is there a pattern?
8. What do you think would happen to cells if they used this spacer in a CRISPR immune response?
9. Why do you think you are able to culture these cells without them dying?

## Part 2: In vitro

1. What is the difference between a guide RNA (gRNA) and a single guide RNA (sgRNA)? You may see the terms used interchangeably, but they are technically different.
2. What are the parts of a sgRNA? What do they each do?
3. Why is RNA so sensitive to degradation compared to DNA?
4. What are some other ways to make double-strand breaks in DNA? How do they compare to Cas9?
5. Why do you need to knock Cas9 off of the cleavage product?

## Part 3: In vivo

1. What are the different parts of the lambda red plasmid responsible for doing?
2. Why do we use WT Cas9 and not the catalytically dead dCas9?
3. Why do we add glucose to the media during the overnight growth, and to the plates tomorrow?

## LabAids specific

1. Why is a non-targeting guide a better negative control than no guide at all?

# Supplementary Teaching Materials cont.

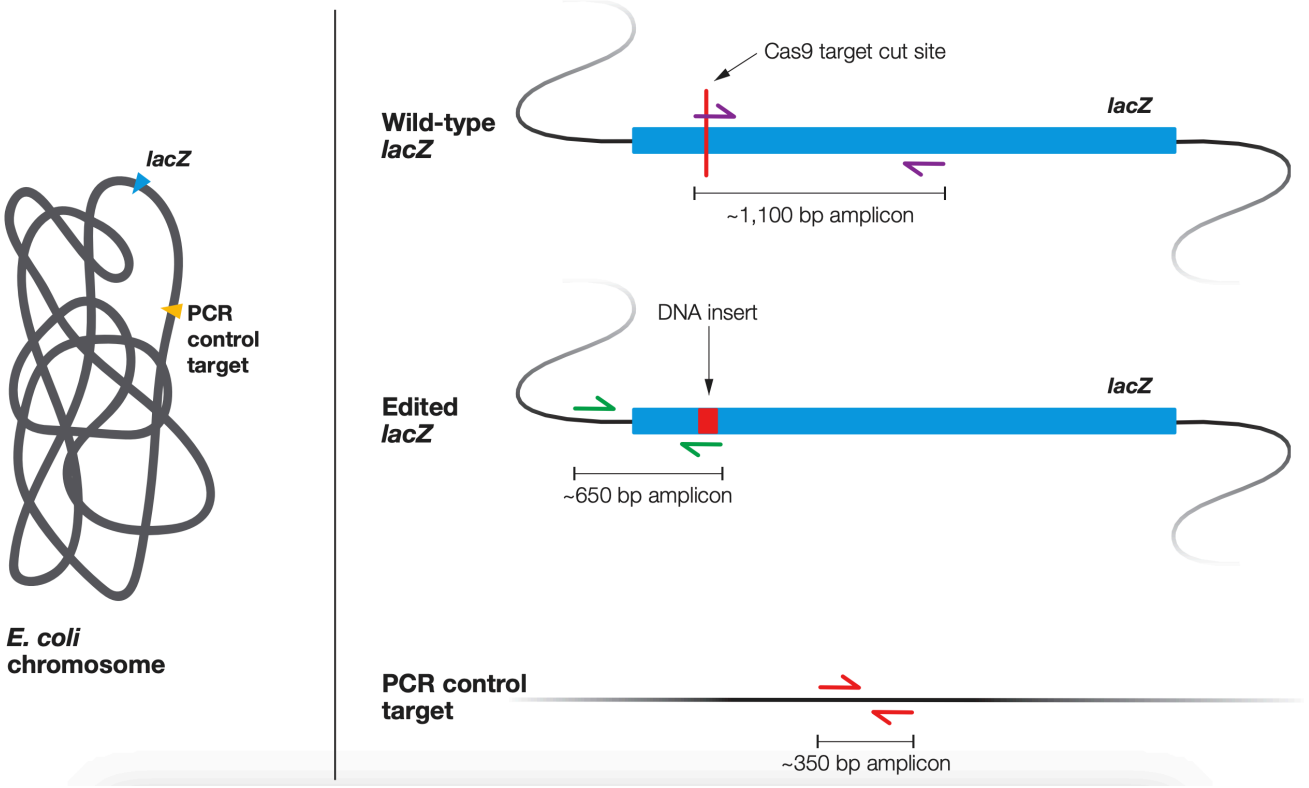
BioRad specific

1. How does the blue/white screening work?

2. Why do all plates need to contain IPTG and X-Gal?

3. One of your plates may have few if any colonies on it. Why do you think that is? Include reasoning for why your evidence supports your claim.

4. In the multiplex PCR, why don't we see the amplicon resulting from the purple reverse primer and green forward primer?



# Additional Resources and Links

CGER Education page
https://www.cger.bio/education

IGI Past Workshop lectures
https://innovativegenomics.org/education/learn-with-us/crispr-workshop-video-collection/

IGI CRISPRpedia (High school, Undergrad level)
https://innovativegenomics.org/crisprpedia/

HHMI Interactive (High school level)
https://www.biointeractive.org/classroom-resources/crispr-cas9-mechanism-applications

Addgene links (Undergrad level)
https://www.addgene.org/guides/crispr/
https://www.addgene.org/educational-resources/ebooks/

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