**CRISPR Gene Editing: Designing the gRNA and Donor Template**

**Piwi Matters Adaptation**

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***Acknowledgements:****This activity was built off of and modified from a CRISPR-based project created by Dr. Jodi Schottenfeld-Roames at Princeton University for MOL350: Laboratory in Molecular Biology.*

**Purpose of Activity:**

This activity is designed to provide students with first-hand experience in designing CRISPR gRNAs and donor templates. Students will learn about the CRISPR/Cas9 bacterial immune system and how it has been modified to facilitate gene editing. Afterwards, students will work through published protocols to design gRNA targets and donor templates with the goal of making a desired mutation in *D. melanogaster*. By modifying this document slightly, the genetic mutation made by the students can be tailored to fit any gene. However, instructors can also use this activity as written to create the Piwi mutation designed by students in the case study*, Piwi Matters*. In *Piwi Matters*, students learn about Piwi’s function and structure to create a novel Piwi mutation.

**Learning Objectives:**

* Describe the immune function of the bacterial CRISPR/Cas9 system.
* Identify and describe the function of each component of CRISPR/Cas9.
* Understand which CRISPR/Cas9 components are necessary for gene editing in eukaryotic cells.
* Design both a gRNA target sequence and a donor template to make a desired mutation.
* Decide the most suitable gRNA target from a list of computer-generated targets.
* Persuade peers to use a specific combination of gRNA target and donor template.
* Evaluate the effectiveness of gRNA target and donor templates to make different mutations.

**Timeline:**

*Instructor Preparation*

Review and adaptation of this document: 4-6 hours

*Course Implementation/Homework*

Initial preview of CRISPR/Cas9 (homework assignment): 1-4 hours

Discussion of CRISPR/Cas9 and constraints (in-class): ~1 hour

Group/Individual design of gRNA sequences and donor templates (in-class): ½-1 hour (additional time may be required as homework)

Submit Piwi mutation, gRNA, and donor template to crowd-sourcing library (in class or as assignment): 15-30 minutes

*Optional Review Panel*

Discussion of review panel and proposal (in-class): 20 minutes

Write ½ page proposal (homework): 30-60 minutes

Peer development of proposal (homework): 30 minutes in class

Peer Presentation (in class): 3-4 minutes per person/group

**Homework:**

Students should review the videos and article below to learn about CRISPR/Cas9. They should pay particular attention to its purpose in bacteria and which components are needed for genetic engineering.

*Videos*

1. Video about CRISPR’s use in gene editing - Mayo Clinic YouTube Video, *CRISPR Explained* <https://www.youtube.com/watch?v=UKbrwPL3wXE>
2. Video about CRISPR’s function in bacteria- Bozeman Science, *What is CRISPR?* <https://www.youtube.com/watch?v=MnYppmstxIs&t=20s>

*Review Article*

1. Thurtle-Schmidt, D.M. and Lo, T. 2018 Biochemistry and Molecular Biology Education. Molecular Biology at the Cutting Edge: A review on CRISPR/CAS9 Gene Editing for Undergraduates. <https://iubmb.onlinelibrary.wiley.com/doi/full/10.1002/bmb.21108>

*Questions*

1. What are three molecules (either RNA or protein) from the *S. pyogenesis* genome that interact with each other to defend the cell against viral infections? What is the function of each component?

Cas9 – Cas9 is a helicase and nuclease. It cuts the DNA of an invading bacteriophage (at a sequence specified by the PAM site and spacer sequence of cRNA).

crRNA – Directs Cas protein to the invading bacteriophage; sequence of RNA complementary to the invading bacteriophage

tracrRNA – Sequence of RNA complementary to the pre-crRNA repeat sequence. tracrRNA forms a double-strand RNA molecule, which is then cleaved by RNAseIII.

1. Briefly describe how bacteria use CRISPR/Cas:
2. during the adaptation phase of immunity

To acquire immunity, the bacteria inserts a fragment of the bacteriophage’s DNA into the CRISPR locus of its genome as a spacer.

1. during the interference phase of immunity

During interference phase, the CRISPR locus is transcribed to create pre-crRNA, which then binds to tracrRNA to form a double-strand RNA complex. The double-strand RNA molecule is cleaved by RNAseIII and then binds with Cas protein to form an active ribonucleoprotein (RNP). The RNP ultimately has the potential to destroy the DNA of an invading bacteriophage.

1. What is a PAM sequence and why is it required for the function of Cas9?

A PAM sequence is a proto adjacent motif (5’ NGG 3’ for Cas9) that must be recognized by Cas9 before a double-strand break can be made.

1. Relative to the PAM site, where does Cas9 create a double-strand break?

Cas9 creates a double-strand break three nucleotides upstream of the PAM site.

1. What are two different ways that eukaryotic cells use to repair DNA after a Cas9 mediated double-strand break occurs?

Non-homologous end-joining

Homologous directed repair

1. What differences between bacteria and eukaryotic cells are important to consider in order to adapt CRISPR/Cas9 for gene editing?

While the bacteria genome encodes all of the CRISPR/Cas9 components (Cas proteins, CRISPR array, tracrRNA), these genes and RNA sequences are not present in eukaryotic cells. (Therefore, eukaryotic cells need to be provided with the sequences for Cas9 and a gRNA. These sequences can be introduced into the nucleus of the eukaryotic cell as transgenes.)

1. What CRISPR/Cas9 components need to be introduced into flies to generate targeted mutations? What is the function of each component?

Cas9 – Recognizes PAM sequences and creates a double-strand break.

sgRNA or gRNA (this is a chimera of the crRNA and tracrRNA) – guides the Cas9 protein to cut a specific site in the DNA.

Donor template – DNA template to introduce a targeted mutation by homologous directed repair (HDR).

1. What steps need to be followed to use CRISPR to create a mutation?
2. Select target and guide, 2) generate and deliver components, 3) verify desired gene edit

**In Class Activity (~20-30 minutes):**

In small groups of 3-5, students review their homework responses. Following the discussion, each group presents a representative answer to one of the questions. In this way, all students walk away with a summary of how CRISPR/Cas9 is used for both bacteria immunity and as a gene editing tool. Depending on the amount of time available, the instructor can select all or a selection of the questions for the class discussion.

*Groups that have finished discussions early, can answer the following questions:*

Why is the CRISPR/Cas9 system able to destroy species of bacteriophages that have already infected the bacteria, but not newly infecting species of bacteriophages? CRISPR/Cas9 system can only be used to defend against bacteriophages that contain a sequence complementary to a spacer sequence present in the bacteria genome’s CRISPR array. Spacer sequences exist for bacteriophage species that have already infected the bacteria (or a previous generation of the bacteria), but need to be acquired for a newly infecting bacteriophage.

Do spacer sequences contain a PAM site? How does this allow the Cas9 protein to distinguish the bacteria genome from viral genome? Spacer sequences in the bacteria’s genome do not contain a PAM site. This prevents the bacteria’s genome from being cut by Cas proteins.

**Transition to designing gRNAs and donor templates (~20-30 minutes):**

Instruct students that this activity is to design the gRNA and single-strand DNA template. Guidelines for designing these components are available in the publication:

 Gratz, S.J. et al 2016. Curr Protoc Mol Biol. CRISPR‐Cas9 Genome Editing in *Drosophila* ***(***[*https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4506758/*](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4506758/)).

As shown in Figure 1 of the publication, students will use the homology directed repair pathway of the fly to edit the sequence of *piwi*. To create the mutation, they would inject flies with: Cas9, gRNA, and a single-strand DNA Donor Vector (ssDNA). However, the Cas9 transgenic flies have already been created. Therefore, these flies only need to be injected with a gRNA target (supplied as a DNA plasmid) and single-strand DNA Donor Vector (ssDNA). The students will be responsible for designing both the gRNA target and the single-strand DNA template.

After describing the objective to the students, use a think-pair-share activity to allow students to brainstorm the constraints needed to 1) design the gRNA and 2) design the donor template. If students are stuck, instructors may want to guide them to page 199 of the review article ([Thurtle-Schmidt, D.M. and Lo, T. 2018](https://iubmb.onlinelibrary.wiley.com/doi/full/10.1002/bmb.21108)).

***gRNA constraints:***

1. 20 nucleotides long
2. PAM sequence should be as close to the desired mutation as possible
3. specific/no off-targets
4. presence of a G or A at the 3’ end of the gRNA target

***donor template constraints****:*

a) contain the desired mutation

b) the original PAM site should be mutated so that it will no longer be a Cas9/gRNA target

c) complementary to the 3’ overhang of DNA

Note: Some classes may generate a list of accurate constraints for both components, while some classes may have more difficulty – particularly on the DNA template constraints. In either case, providing time for students to predict the constraints will prepare them to build connections between the Thurtle-Schmidt, D.M. review and Gratz, S.J. published protocol.

**gRNA target design**

To design an ideal gRNA target sequence, students will follow steps published in the paper: Gratz, S.J. et al 2016. Current Protocols in Molecular Biology. CRISPR‐Cas9 Genome Editing in Drosophila <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4506758/>. In this paper, students should read the section titled, “Target Site Selection“ and list at least two constraints to consider when choosing the gRNA target. Answers are listed below.

1. gRNA target needs to create a double-strand break (DSB) near your desired mutation.
2. gRNA target needs to be unique.
3. gRNA must exactly match the genome sequence.
4. gRNA must be adjacent to a PAM sequence.

With these constraints in mind, students will design a gRNA sequence by following steps 6-11 of Gratz, S.J. et al 2016. Students will start at step 6 because steps 1-5 describe the methods to determine the specific sequence of *piwi* in Cas9 transgenic flies. Instead, students will refer to the sequence of NCBI Accession # AF104355: <https://www.ncbi.nlm.nih.gov/nuccore/AF104355>.

While designing the gRNA target and DNA template, students should be comfortable explaining why they have made their decisions. These rationales can be used in the optional activity of running a mock review panel. After the panel, students vote on the combination of a *piwi* mutation, gRNA, and DNA template that most convincingly leads to the predicted disruption of Piwi function.

**Questions to be included on Student Worksheet**

**Enter the Sequence of your gRNA:**

**Briefly explain why you chose this gRNA sequence:**

**Single-strand DNA template design**

To design a short single-strand DNA template, students will follow steps published in the paper: Gratz, S.J. et al 2016. Current Protocols in Molecular Biology. CRISPR‐Cas9 Genome Editing in Drosophila <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4506758/>. In this paper, students should read the section titled, ”Alternate Protocol 1 HDR with single-stranded DNA donors“ and list four constraints to consider when choosing your gRNA target. Answers are listed below.

1. The optimal distance between the Cas9 cute site and the desired mutation is 1-4 nucleotides.
2. Each homology arm should be 75-100 nucleotides long.
3. The ssDNA be complementary to the free 3' end
4. PAM sites should be mutated so that the donor template is not cut by Cas9

**Questions to be included on Student Worksheet**

**Enter the Sequence of your single-strand DNA template design. Mark any mutated nucleotides and denote whether the mutations 1) create your desired mutation or 2) mutate the PAM site.**

**Briefly explain the strengths and pitfalls of your template:**

**Class Review Panel – Optional Class Activity**

The mock grant proposal offers an opportunity for students to engage in persuasive communication. For this activity, inform students that effective persuasion requires them to clearly explain their rationale and effectively communicate how the mutation will be generated with the given gRNA target and donor template.

Inform students that there will be a class review panel to decide which mutation should be made. To prepare for the review panel, students should complete a homework assignment of writing a ½ page discussion answering the questions:

1. What mutation are you creating in Piwi?
2. Why are you creating this mutation? In other words – what function of Piwi will be lost in this mutant?
3. What gRNA sequence are you using? Why?
4. What ssDNA template sequence are you using? Why?

Depending on the time available and the size of the class, students can work individually or collaboratively to transform their written responses into 2-4 minute presentations. The short presentations will then be presented to the class. After presentations, students vote on which mutation, gRNA, and donor template are most likely to disrupt the proposed function of Piwi. The presenters with the most votes win a snack!

As a self-reflection of their persuasive communication, students should write a 1-minute essay to answer the question:

What strategies could be used to further persuade others to create your Piwi mutation?

**Submitting student mutations, gRNA sequences, and donor templates to our database:**

The final step of this activity is for students to submit their gRNA target and donor template sequences to a crowd-sourcing library. This library will be used to generate flies with new Piwi mutations.

To contribute responses, students should fill out the form at: <https://forms.gle/FLcSkE56iA8j9HhP9>

Copies of the submitted responses can be emailed to the instructors upon request. Email requests to Laurel Lorenz: llorenz@princeton.edu.

**References**

Gratz, S. J., C. D. Rubinstein, M. M. Harrison, J. Wildonger and K. M. O'Connor-Giles, 2015 CRISPR-Cas9 Genome Editing in Drosophila. Curr Protoc Mol Biol 111: 31 32 31-31 32 20.

*Published protocol to design gRNA targets sequences and donor templates.*

Lorenz, L., and S. Dutta, 2020. Piwi Matters. Molecular CaseNet Faculty Mentoring Network, Qubes Educational Resources.

*A structure/function case study for students to design novel Piwi mutations.*

Port, F., H. M. Chen, T. Lee and S. L. Bullock, 2014 Optimized CRISPR/Cas tools for efficient germline and somatic genome engineering in Drosophila. Proc Natl Acad Sci U S A 111: E2967-2976.

*This reference is particularly helpful as a reference for the rationale of using different vectors for gRNAs.*

Zimmer, C. T., W. T. Garrood, A. M. Puinean, M. Eckel-Zimmer, M. S. Williamson et al., 2016 A CRISPR/Cas9 mediated point mutation in the alpha 6 subunit of the nicotinic acetylcholine receptor confers resistance to spinosad in Drosophila melanogaster. Insect Biochem Mol Biol 73: 62-69.

*This reference is a proof of concept to generate point mutations in Drosophila. Methods are particularly helpful to understand the design of gRNA targets and donor sequences.*