Using fluorescent proteins as reporters of CRISPR-Cas9 activity

Objective: Use freeze-dried, cell-free (FD-CF) reactions and a CRISPR-Cas9 system to determine which fluorescent protein DNA sequence (target DNA) is targeted by a mystery guide RNA.

Pre-lab Activity:

Review the components of the CRISPR-Cas9 system. What role does each component play in the activity of CRISPR-Cas9? Describe the role of each component in the table below:

CRISPR-Cas9 System	
Component	**What role does this component play in CRISPR-Cas9 activity?**
Cas9	
Guide RNA	
PAM	
Target DNA	

1) How will you know if Cas9 was active?

2) If the Cas9 guide RNA works (binds) your target DNA, what do you expect to observe?

3) If the Cas9 guide RNA does not work, what do you expect to observe?

Procedure:

Using fluorescent proteins as reporters of CRISPR-Cas9 activity lab

Materials needed:

- FD-CF reactions in PCR tube strips (4 reactions per group)
- Large-scale (15 µL) FD-CF reactions in microcentrifuge tubes (5 µL of reaction mixture needed per group)
- 13.33 ng/µL Cas9 plasmid DNA stock solution (provided)
- 13.33 ng/µL fluorescent protein plasmid DNA stock solution (one each of mCherry, mRFP1, dTomato, mOrange,YPet, and sfGFP plasmid DNA provided)
- 33.33 ng/µL gRNA plasmid DNA stock solution (provided)
- Nuclease-free water (provided)
- Sterile pipet tips and micropipettes
- PCR thermocycler OR BioBits™ portable incubator
- Blue light imager OR BioBits™ portable imager

DAY 0

1. Rehydrate large-scale FD-CF reactions by adding 15 µL of Cas9 plasmid DNA stock solution to the FD-CF reactions in microcentrifuge tubes. Close the lid and gently flick the side of the tube to dissolve the freeze-dried CFPS pellet. Reactions can be incubated in the BioBits™ portable incubator or a PCR thermocycler for 20-24 hours at 30°C or on the benchtop at room temperature for 24-48 hours. These reactions will pre-express the Cas9 protein that you will use in your experiment. <u>You will need to prepare 5 µL of Cas9 reaction mix per group.</u>
   This step can be done by the instructor or students.

DAY 1

2. Obtain one of the fluorescent protein plasmid stocks from your instructor. Depending on class size, each group can test the activity of the mystery gRNA on one or multiple of the fluorescent proteins. The class' combined data should test the ability of the gRNA to target each of the 6 fluorescent proteins.
3. Label a strip of four FD-CF reactions with the name of your fluorescent protein plasmid. Label two of these tubes with "-" and two with "+" to indicate which tubes will include gRNA plasmid.
4. Add the appropriate amounts of Cas9 reaction mix, gRNA plasmid stock, and water (refer to the table below) to each of the FD-CF reactions.
    a. Add water first, then fluorescent protein plasmid, then Cas9, then gRNA plasmid.
    b. You can use the same pipet tip for each water addition as long as you do not touch any surface other than the inside of the tubes! For pipetting Cas9, fluorescent protein plasmid, and gRNA plasmid you'll need to switch pipet tips.
    c. *Be careful to avoid touching the white pellet at the bottom of the tubes!*

Tube	Water (µL)	Fluorescent protein plasmid (µL)	Cas9 FD-CF reaction (µL)	gRNA plasmid (µL)
- gRNA	4	1	1	0
+ gRNA	3	1	1	1

5. Seal your FD-CF reactions using an 8-strip PCR tube lid. Gently flick the side of the PCR tubes to dissolve the pellet in the Cas9/DNA/water mixture that you added.
6. Allow the reactions to incubate until the next time you come to class. Reactions can be incubated in a PCR thermocycler for 20-24 hours at 30°C or on the benchtop at room temperature for 24-48 hours. Get excited!

DAY 2

7. Obtain your group's samples from your teacher. Place your strip of reactions in front of a piece of white paper and record your observations.
8. Take a picture of your reactions under blue light (using the BioBits™ blue light imager or other blue light source) and record your observations.
9. Quantify fluorescence produced in each reaction with ImageJ, using the BioBits™ Health ImageJ tutorial. Record the relative fluorescence unit (RFU) values you measure in ImageJ in the table below. Consult with your classmates who tested other fluorescent protein targets to complete the data table for all 6 fluorescent proteins.
10. Calculate the average and standard error of the RFU values you measured using Excel. Record these values in the table below.
11. Plot the average RFU values you calculated as a function of gRNA addition for each fluorescent protein target. Choose an appropriate graph type (bar, scatter, etc.) to display your results. Add error bars showing the standard error of your measurements.
12. *Optional: Use the "t-test: Paired Two Sample for Means" function in Excel to determine if the addition of gRNA results in statistically significant differences in fluorescence for each fluorescent protein target. The following tutorial can help you get started:* https://www.rwu.edu/sites/default/files/downloads/fcas/mns/running_a_t-test_in_excel.pdf

Data:

Fluorescent protein: mCherry				
Guide RNA?	RFU reaction 1	RFU reaction 2	Average RFU	Std Error RFU
-				
+				

Fluorescent protein: mRFP1				
Guide RNA?	RFU reaction 1	RFU reaction 2	Average RFU	Std Error RFU
-				
+				

Fluorescent protein: dTomato				
Guide RNA?	RFU reaction 1	RFU reaction 2	Average RFU	Std Error RFU
-				
+				

Fluorescent protein: mOrange				
Guide RNA?	RFU reaction 1	RFU reaction 2	Average RFU	Std Error RFU
-				
+				

Fluorescent protein: YPet				
Guide RNA?	RFU reaction 1	RFU reaction 2	Average RFU	Std Error RFU
-				
+				

Fluorescent protein: sfGFP				
Guide RNA?	RFU reaction 1	RFU reaction 2	Average RFU	Std Error RFU
-				
+				

Post-lab Analysis:

Which fluorescent protein gene did the gRNA target? How do you know?

Answer Key:

CRISPR-Cas9 System	
Components/Acting Molecules:	**What do you expect this molecule to do? What will happen?**
Cas9	Enzyme that can cut DNA. If guided by gRNA to a specific target cute site, it will cut the DNA at that site.
Guide RNA	A synthetic piece of RNA that consists of a scaffold that can bind Cas9 and a spacer that can be designed to target any DNA sequence. Using this setup, the gRNA guides the Cas9 to the target cut site so the Cas9 enzyme can cut that site.
PAM	Protospacer Adjacent Motif – the target cut site needs to be immediately adjacent to the relevant PAM sequence. The PAM sequence allows for Cas9 to bind and cleave the target DNA sequence.
Target DNA	The DNA molecule that contains the target cut site. The gRNA will bind to this DNA molecule at a specific site for Cas9 to cut there.
Cut sites	The cut site is the target where we want the Cas9 to cut. The spacer on the gRNA will be complementary to this cut site in order to guide the Cas9 to the target cut site on the target DNA.

1) How will you know if Cas9 was effective? The CRISPR-Cas9 system will target one of the fluorescent-protein-encoding DNA sequences. If Cas9 is effective in cutting at the target cut site, then the DNA sequence will be split in two and therefore will not be able to undergo transcription and translation to make the fluorescent protein, leading to no observed fluorescence.

2) If the Cas9 guide RNA works (binds) your target DNA, what do you expect to see?
Once the Cas9 guide RNA binds to the target DNA, the Cas9 will cut the target DNA at that specific cut site. Because the DNA is now cut, transcription cannot happen to make the RNA, and then the reporter protein cannot translated, and thus no fluorescence will be observed.

3) If the Cas9 guide RNA does not work, what do you expect will happen? If the Cas9 guide RNA does not bind, then the Cas9 will not cut the DNA. If the DNA remains uncut, it will be able to undergo transcription and translation to create its encoded fluorescent protein, which we can observe.

Note: This is what is expected assuming that everything went correctly. If student results do not match this table, encourage a discussion where different groups can compare their results and come up with hypotheses why their results were different.

	Guide RNA?	
Target Protein	**-**	**+**
mCherry	Red fluorescence	Red fluorescence (same as –gRNA)
mRFP1	Red fluorescence	Decreased or no red fluorescence (compared to –gRNA)
dTomato	Orange fluorescence	Orange fluorescence (same as –gRNA)
mOrange	Orange-yellow fluorescence	Orange-yellow fluorescence (same as –gRNA)
YPet	Yellow fluorescence	Yellow fluorescence (same as –gRNA)
sfGFP	Green fluorescence	Green fluorescence (same as –gRNA)

Which fluorescent protein gene did the gRNA target? How do you know? The gRNA is designed to target the DNA that encodes for mRFP1. This is because for all other proteins, we did not see a difference in protein expression between the reactions where we didn't add gRNA and the reactions where we did add gRNA. This means the CRISPR-Cas9 system did not bind and cleave the DNA because the DNA was still able to be used in transcription and translation to create the fluorescent protein. On the other hand, the mRFP1 reactions show a decrease in fluorescence if the gRNA was added. This means that the CRISPR-Cas9 system did bind and cleave the DNA, causing it unable to be used in transcription and translation to create the mRFP1 fluorescent protein.

Teacher Notes:

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