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# Scarless introduction of point mutations in mammalian cells with S. pyogenes Cas9

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ABSTRACT

This protocol outlines the steps to edit single point mutations into cells in a scarless manner. Uses single-stranded DNA repair templates and the S. pyogenes CRISPR/Cas9 system.

Using a short single-stranded DNA repair template is advantageous because it is easy to order synthetically and it cannot be randomly integrated into other double-strand breaks in the cell. Furthermore, this protocol is "scarless" - that is, nothing is changed to the genome other than the desired changes. However, it may be easier to edit genes using a selectable marker, especially when knocking out a gene. This protocol is ideal when trying to make genetic changes with minimal perturbations to the gene locus and surroundings.

Note that repair using a single-stranded short repair template (SSTR) uses a different DNA repair pathway than homology-directed repair from long DNA repair templates.

Citations:
image 1: synthego.com- verify your guide design tool

## Workflow

1 The workflow for gene editing with the CRISPR/Cas9 system follows these basic steps:

1. Designing appropriate sgRNAs (recruits Cas9 enzyme to DNA). Cas9 bound to the sgRNA is referred to as the "RNP" (ribonucleoprotein)
2. Nucleofecting RNPs to test cutting efficiency for each sgRNA
3. Designing appropriate single stranded repair templates (with mutation)- it is important to wait until after you have chosen the best sgRNA to desgin your repair template, as each template is designed based on the sgRNA
4. Designing appropriate PCR primers flanking your mutation site for genotyping
5. Selecting the sgRNA with the highest cutting efficiency and nucleofecting cells with both the repair template and RNP
6. Diluting nucleofected cells in a 96 well plate to generate single cell clones
7. Analyzing single cell clones for Knock-in efficiency of the repair template

## Designing sgRNAs

2 In order to identify a guide with the highest cutting efficiency at the mutation site, it is good practice to design 3-4 sgRNAs.

- First, download the genomic DNA transcript of your gene on any platform. For the purposes of this protocol, Benchling will be used. It is important to use genomic DNA and not cDNA or an mRNA sequence, since introns are in genomic DNA but not in cDNA or mRNA sequences.
- Locate the mutation site of interest in the gDNA transcript.
- Cas9 introduces a double stranded break in DNA. The closer the double stranded break is to the mutation site, the better. Ideally the cut will be within 10 nt of the desired mutation.
- Locate the PAM (protospacer adjacent motif) sequence. The PAM sequence is a three nucleotide sequence that recruits Cas9 to the DNA. The PAM sequence for S. pyogenes Cas9 is NGG (5'-->3') and the double stranded break will be three nucleotides 5' of the PAM

sequence. Ensure to look both on the + and - strand of the transcript for PAM sequences (the - strand PAM is CCN and the cut site is on the opposite side compared to NGG PAMs).

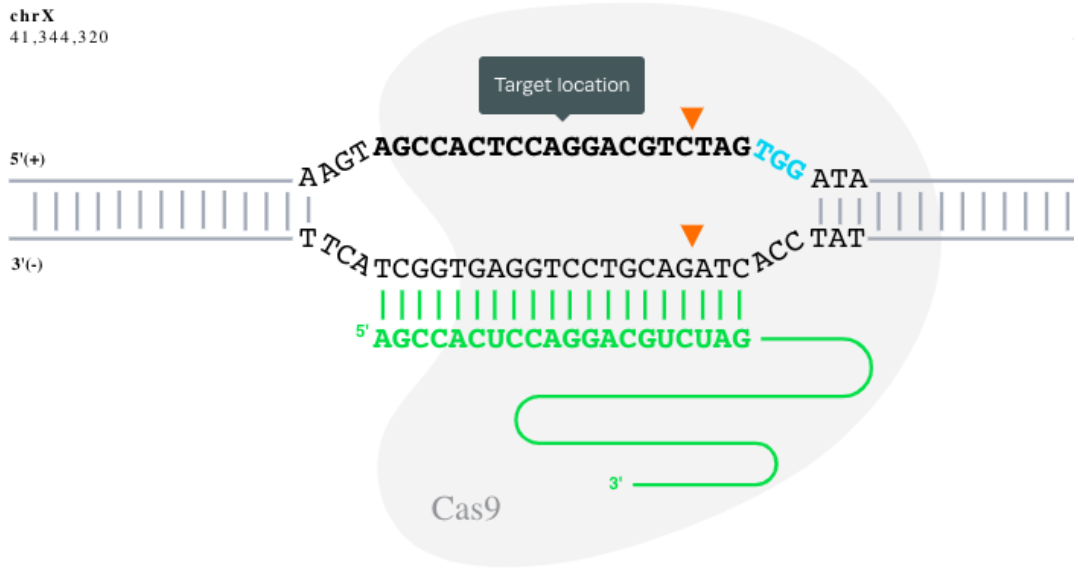
- Example: CATTTGTTAGTAGCCACT**CCA**GGA||CGTCTAGTGGATATGATGGAA
- Your protospacer/target sequence will be 20 nucleotides long adjacent to the PAM sequence.
- Example: CATTTGTTAGTAGCCACT**CCA** *GGA||CGTCTAGTGGATATGAT*GGAA (protospacer italicized)
- Example other strand: ATTTGTTAGTAGCCACTCC*AGGACGTCTAGTGGATA||TGA***TGG**AA
- Once you have selected 3-4 sgRNA sequences, visit synthego.com and verify your guide design.
- In vitro transcribe your guides or order them from Synthego or elsewhere.

## Designing repair templates

3 Single stranded repair templates need to be desgined appropriately, in order to maximize Knock-in.

- Your repair template will be exactly 120 nts and assymetric (90:30nts), where the assymetricity depends on the target strand; (+) strand or (-).
- First, identify your target strand. Your target strand is:
  -opposite your PAM sequence and target sequence.
  - the strand that hybridizes with the sgRNA.

Example: In this case, your target strand is on the (-) strand.




- Your repair template is a modification of the target strand. Therefore, all mutations will be made on the target strand.
- In regards to the assymetricity, 30 nts of your repair template should bind the 5' end of the double stranded break, and 90 nts should bind the 3' end.
- Make appropriate changes to your repair template in order to reflect your mutation. Ensure to make a silent mutation to change the PAM sequence, in order to avoid Cas9 cutting of your repair template. If a silent mutation to change the PAM is not possible, introduce 2-3 silent mutations in your target sequence to prevent your sgRNA from recognizing your repair template. Mismatches closer to the PAM are more effective at preventing re-cutting.
- Example:
  -In the case shown above, the 3' end of the repair template will be 30 nts and the 5' end of the repair template will be 90 nts.

## Primers flanking mutation site

4 To get the best PCR product flanking your mutation site:

- Design 4-5 sets of primers flanking your mutation site, that give you a product size of approximately 500 base pairs.
- Ensure your product size is greater than 120 bps to avoid a PCR product of your repair template.
- A useful tool in designing primers is NCBI primer blast.
- Once your have received your primer sets, run a gradient PCR with genomic DNA for all your primers. (Use a DNA polymerase of your choice).
- Run your PCR products on an agarose gel and analyze the best primer set and annealing temperature to use. Ideally there will be a single well-defined band.

## Nucleofecting sgRNAs

## Nucleofecting sgRNAs

5 Once your sgRNAs have arrived, you can nucleofect them into the cells of your choice to measure cutting efficiency.
- Before nucleofection, resuspend your sgRNAs to a concentration of 200uM in TE buffer.
- Follow https://www.protocols.io/view/cas9-sgrna-ribonucleoprotein-nucleofection-using-l-2ijgccn or any other version of this nucleofection protocol that is best suited for your cell type.
- For N2A cells use program: Neuro2a-HE for 4D Lonza nucleofector in SF cell line nucleofector solution.
- For iPSCs use program: CB-150 for 4D Lonza nucleofector in P3 Primary nucleofector solution. (ensure you use Rock inhibitor as needed- during centrifugation of cells and plating of cells)
- Once your nucleofection is complete, allow two days for your cells to recover.
- Extract genomic DNA from your nucleofected cells.
- Run a PCR with the appropriate primer set using the extracted gDNA.
- Purify your PCR product and send the product for sequencing.
- Once your sequencing data is available, analyze the results with Synthego's ICE analysis tool.
- The sgRNA with the highest ICE score is the best guide to use.

## Nucleofection with repair template

6 Once you have selected the best guide to use, you can now nucleofect with your repair template.
- Resuspend your repair template to a concentration of 100uM in TE buffer.
- Use the same nucleofection protocol from above to nucleofect cells with your repair template.
- Once your cells have been nucleofected, give them two days to grow.
- Extract genomic DNA from some cells, freeze down some cells, and dilute others as below.
- PCR the gDNA, purify the PCR product, and send the product for sequencing.
- Use Synthego's Knock-in efficiency tool to analyze the KI in the pool of cells. High-efficiency knock-in will be around 30-50% of cells, while other times it is much lower. Use the pool KI efficiency to see if it is worth single-cell cloning.
- If the KI efficiency is high enough, serial dilute cells in a 96 well plate to get single cell clones using this protocol: https://www.corning.com/catalog/cls/documents/protocols/Single_cell_cloning_protocol.pdf

## Selecting single cell clones and analyzing them

7 Once diluted, give your cells one to two weeks before screening the 96 well plates for single cell clones.
Follow the guidelines on the dilution protocol in order to learn how to pick single cell clones.
- Once you have selected a few single cell clones, replate them into a 24 well plate.
- Once confluent, extract genomic DNA from some cells and propagate the rest.
- PCR with this gDNA, purify the PCR product, and send the product for sequencing.
- Use synthego's KI analysis tool to analyze the KI efficiency in each cell line.
- If a dilution does not work to separate your cells (especially for "clumpy" cells), you can also use FACS to sort single cells into a 96 well plate.