

CRISPR-Cas9 genome editing by RNP injection

By Shanon Brady, Daehan Lee, Clay Dilks, Emily Koury, and Erik Andersen
Updated November, 2021

This protocol describes the steps to make a CRISPR-Cas9 RNP mixture for microinjection and the steps after microinjection to screen for edits. Microinjection is a separate protocol. Please see the **ordering information guide** document for Andersen lab specific ordering instructions for the reagents below. For a quick video explaining the basic process, please follow [this link](#). Note that there are key differences in the video to this protocol and you should follow this protocol explicitly where they differ.

Reagents:

sgRNA:

Design your guides near your targeted edit site using the instructions below. Order sgRNAs from Synthego and resuspend with provided TE to 100 μ M. Store at -20°C

target repair oligo:

Design your repair oligo using the instructions below. Order as 4 nmol Ultramer DNA oligo from IDT and resuspend to 100 μ M in nuclease free water. Store at -20°C.

dpy-10 sgRNA sequence: GCUACCAUAGGCACCACGAG

Order from Synthego and resuspend with provided TE to 20 μ M. Aliquot and store at -20°C

dpy-10 repair oligo sequence:

CACTTGAACTTCAATACGGCAAGATGAGAATGACTGGAAACCGTACCGCATGCGGTGCCTATGGT
AGCGGAGCTTCACATGGCTTCAGACCAACAGCCTAT

Order using the above sequence as a 4 nmol Ultramer DNA oligo from IDT and resuspend to 10 μ M in nuclease free water. Aliquot and store at -20°C.

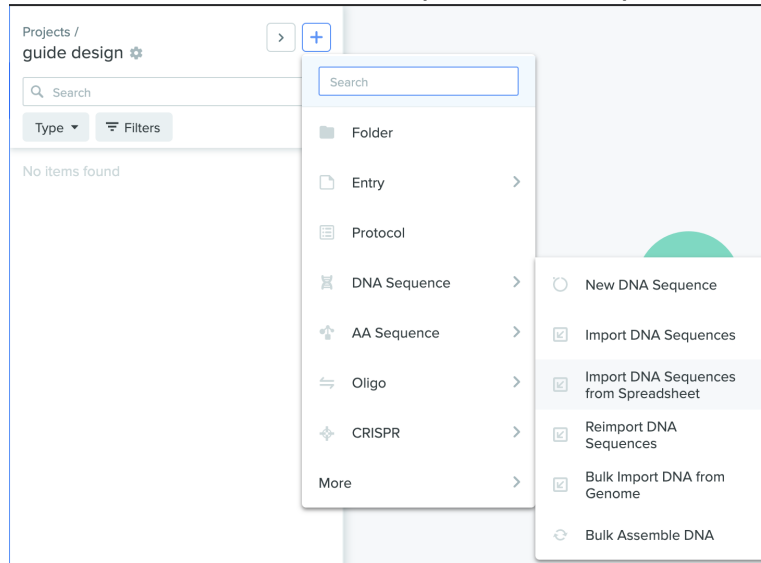
Cas9:

40 μ M Cas9-NSL purified protein obtained from MacroLab Facility at the University of California, Berkeley. This order comes in 10 μ L aliquots that are marked with each use and discarded after four freeze/thaws.

Designing guides on Benchling

We use Benchling to find guides and look for the highest possible on-target and off-target scores. Below is a step-by-step guide using *ben-1* as an example. For homology-directed repair, design one guide (along with a repair oligo, see next section). For a large deletion using non-homologous end joining, design two guides flanking the region targeted for deletion.

1. In Benchling, click the + icon. Then select DNA sequence and import DNA sequences.



2. Click search external databases and enter the Wormbase ID for your targeted gene. Then, specify the most recent genome available (currently WBcel235) and enter additional upstream and downstream base pairs (bp) if desired (this extra sequence can be useful when making a large deletion or for external primers near either end of the gene). Click import.


Convert Files Search External Databases Select Chromosomal Region Input Raw Sequence

Example searches:

- <https://www.addgene.org/browse/sequence/327820/> (Addgene URL)
- BRCA2 (Gene Name)
- M62653 (NCBI Accession)
- ENSMUSG0000041147 (ENSEMBL ID)
- BBa_E0040 (Registry of Standard Biological Parts)
- JPUB_001430 (JBEI Public Registry)

Import multiple sequences at once by entering space-separated or comma-separated accession numbers.

WBGene00000248 Search

 **Genome** WS220 (ce10, Caenorhabditis e)
 Don't see the genome you're looking for? We may be able to import it, [just ask](#).

Gene WBGene00000248 (C54C6.2)

Location chrIII 3,538,280—3,541,615 (-)
 Import in sense orientation

Transcript C54C6.2 (C54C6.2)
 Import cDNA only

Upstream bp 0

Downstream bp 0

Import As C54C6.2 (C54C6.2)

Save To

3. Select the region containing the desired edit and click the CRISPR icon on the right side of the screen. Then, click the design and analyze guides button.

The screenshot shows a web-based CRISPR design tool interface. At the top, there are tabs for different sequences: 'ben-1_for_em...', 'NHR-176', 'cyp-35D1', 'Katie's ttTI5605 ...', 'pUC19', and 'Katie's IDT ttTI56...'. Below the tabs are navigation options: 'SEQUENCE MAP', 'LINEAR MAP', 'DESCRIPTION', and 'METADATA'. A 'Share' button is visible in the top right. The main area displays a DNA sequence with a protein translation below it. The sequence is color-coded by amino acids. A vertical line indicates a target site. Below the sequence, several restriction enzymes are listed: BsgI, PshAI, DraIII, BsaAI, PmlI, BanII, EaeI, and CspCI. A 'CRISPR' menu is open, showing options: 'DESIGN AND ANALYZE GUIDES', 'Saved Guide Analyses', and 'DESIGN HR TEMPLATE (SSODN)'. The background shows a scale from 2,060 to 2,160 and another from 2,180 to 2,260.

4. Select the desired design type (single guide), guide length (20 bp), genome, and 3'-NGG PAM site.

The screenshot shows a dialog box titled 'Design CRISPR Guides: Guide parameters'. It has a close button (X) in the top right corner. The dialog contains the following settings:

- Design Type:**
 - Single guide (Wild-type Cas9, single gRNA (higher efficiency))
 - Paired guides (Double Cas9 nickase, two gRNAs (lower off-target effects))
 - Guides for "base editing" (Komor et al., 2016) (C → T (or G → A) substitution, no dsDNA breaks)
- Guide Length:** 20
- Genome:** WBcel235 (Caenorhabditis elegans)
 - Don't see the genome you're looking for? We may be able to import it, [just ask.](#)
- PAM:** NGG (SpCas9, 3' side)

At the bottom right, there is a link for 'Advanced Settings'. At the bottom left, there is a checkbox 'Save these as my default CRISPR settings' which is checked. A 'Finish' button is located at the bottom right.

5. The results page contains potential guides for this region. Attempt to find a guide that is both close to your edit and has the highest on-target and the highest off-target scores. A bad score 10 bp away from edit is generally better than a good score 30 bp away. Do not choose a guide more than 30 bp away.

Target Region: 1676 1725

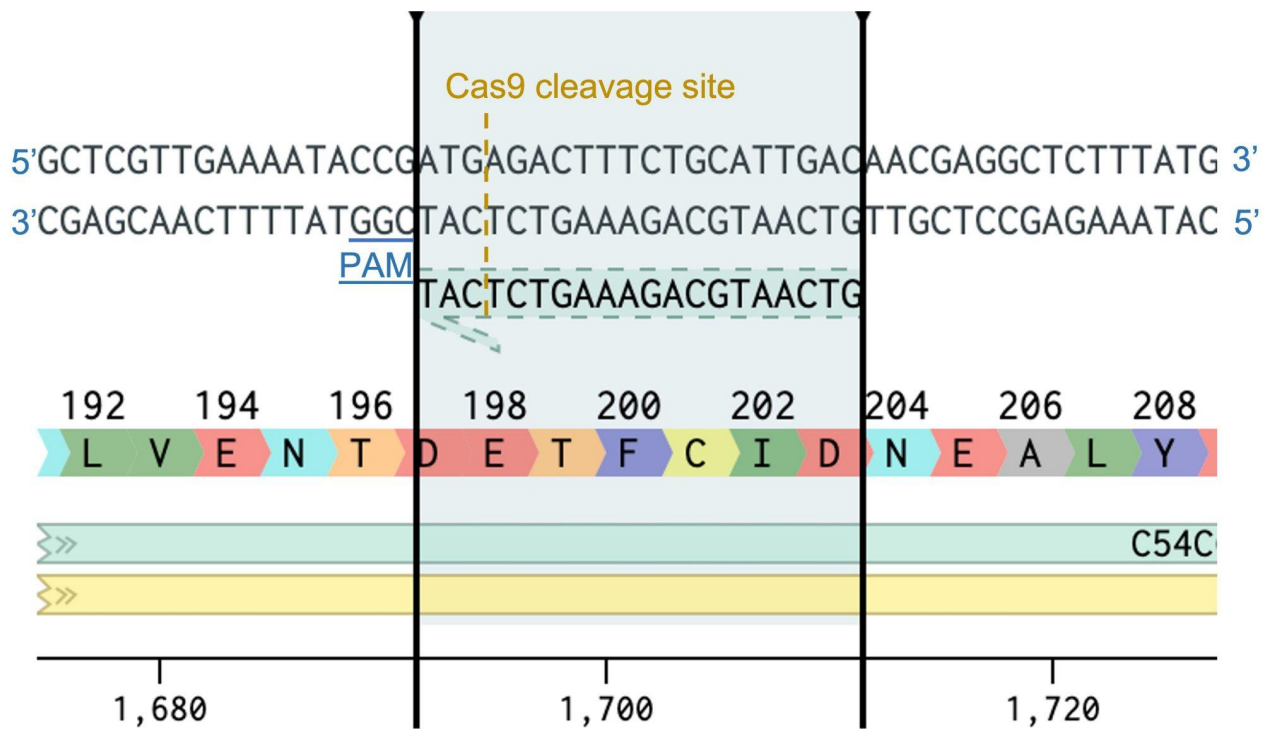
Start	End	Annotations	Genome Region
1676	1725		No region set

You don't have a genome region set above, so scores may not match other sites such as crispr.mit.edu. Benchling uses the genome region to locate your target region and to ignore potential off-target sites in that part of the gen

Export Save Assemble 0 selected 1-2 of 2

Position	Strand	Guide Sequence	PAM	On-Target Score	Off-Target Score
1695	-	GTCAATGCAGAAAGTCTCAT	CGG	58.3	49.4
1713	+	GACTTTCTGCATTGACAAACG	AGG	65.8	48.1

6. Click the checkbox on the guide and go back to the sequence by selecting the tab at the top. Your guide will now appear along with the sequence. The double-strand break (DSB) cut site is 3 bp 5' from the 3' end of your guide (where the PAM site should start).



7. Check your guide + PAM site sequence against the reference genome with BLASTn using the [WormBase](https://www.ncbi.nlm.nih.gov/blast/) website to check for multiple cut sites. If there are multiple potential cut sites (*i.e.*, close identity to the 3' end of the guide + the PAM site, in more locations than your target site), redesign your guide.

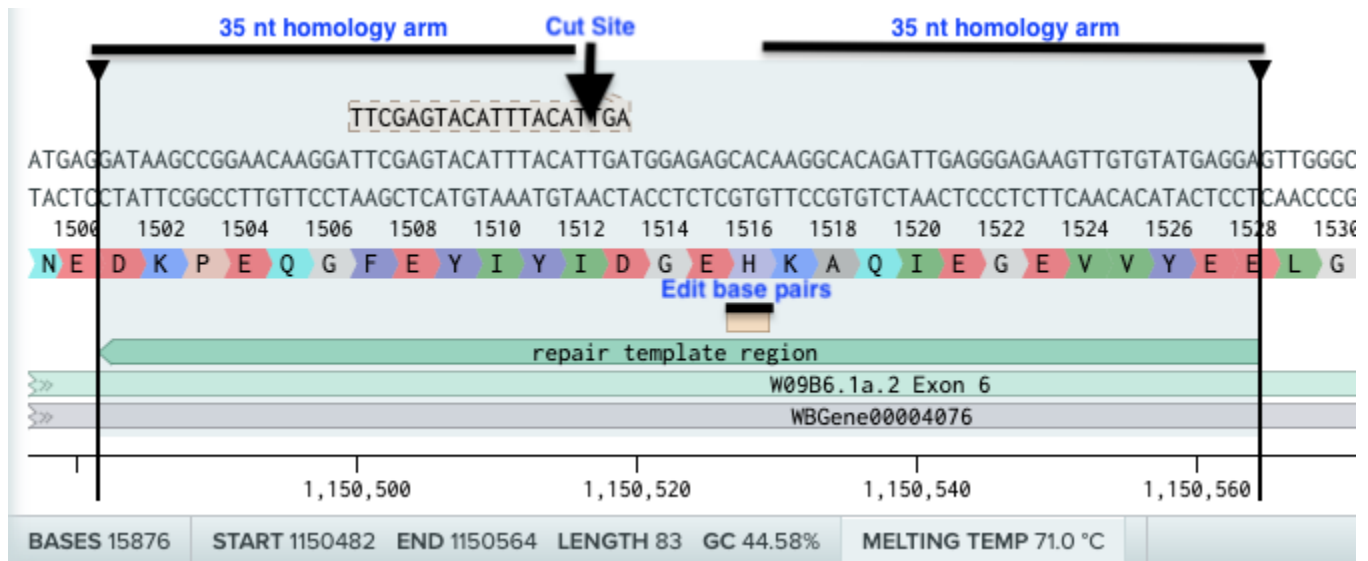
Repair oligos on Benchling

We use Benchling to design repair oligos as shown below:

1. After designing your sgRNA, locate the cut site and use this sequence to determine your repair oligo sequence using the recommendations below. The cut site occurs upstream from the 3' end of your guide/PAM site, between the 4th nucleotide and 3rd nucleotide from the 3' end of your guide.

Repair oligos should have:

(i) opposite stranded-ness as the sgRNA, and (ii) homology arms 35-40 bp on both sides of the DSB and outside of the edit. The region between the cut site and your edit are not part of either homology arm and should be no further than 30 bp away. The repair oligo no longer needs to have a synonymous change in the PAM site or two synonymous changes within the crRNA region though this change could be considered later if your edit proves to be difficult.



2. Select the entire region and annotate your file. You may want to make a copy of your file in Benchling with your edit in the sequence as well.

3. Create a repair sequence in LabGuru based on that selection, changing the sequence to the edit. Your repair oligo sequence should match the desired sequence for the edit you wish to make.

4. Order as 4 nmol Ultramer DNA oligos from IDT and resuspend to 100 μ M in nuclease free water. Store at -20°C.

Designing screening primers

Design and test screening primers before attempting genome edits

Large Deletion

For a large deletion, design and test a pair of primers flanking 100-500 bp away on either side of the cut site for each guide for a product size of 200-1000 bp in size. Also test the outer primers from each set with each other to have a set that spans the region of deletion. This product might be too large to reliably amplify in the unedited strain lysis but should create a band once the deletion is created and the product is small enough to amplify. When screening for your deletion, PCR the single worm lysis (SWL) from all candidate edited strains with one or two of the flanking pairs as well as the deletion spanning pair to test for both the loss of the internal PCR product and the gain of a band for the deletion spanning pair. Sequence your successful edits to confirm and characterize your new strains.

Sequence change without a large change in size

Design and test primers flanking at least 100 bp away on either side of the edit. For a cleaner Sanger sequence, design primers further away to make a product 400-1000 bp in size and design a second primer 200-100 bp from the edit to sequence the PCR product. Sequence the background genotypes to ensure your sequencing will be efficient.

Assembling your CRISPR-Cas9 RNP Mix

1. Shown below are master mix recipes for either homologous repair using an oligonucleotide or generation of a deletion allele using two Cas9 RNPs. Both use the *dpy-10* co-CRISPR technique. The total volume of one mixture is 20 μ L, which is then divided into four single-use 5 μ L mixtures with one used immediately. The frozen RNA mixture aliquots might have reduced efficiency than the unfrozen fresh mix but have still worked well in our hands.

Homologous repair CRISPR mix

Reagent	Amount in μ L
sgRNA <i>dpy-10</i> [20 μ M]	1.0
target edit sgRNA [100 μ M]	1.2
<i>dpy-10</i> repair oligo [10 μ M]	1.0
target repair oligo [100 μ M]	1.0
Cas9	1.64
dH ₂ O	14.16

Deletion CRISPR mix

Reagent	Amount in μ L
sgRNA <i>dpy-10</i> [20 μ M]	1.0
target sgRNA #1 [100 μ M]	0.6
target sgRNA #2 [100 μ M]	0.6
<i>dpy-10</i> repair oligo [10 μ M]	1.0
Cas9	1.64
dH ₂ O	15.16

2. Make a mix using the recipe above. When thawing a Cas9 aliquot, mark the aliquot tube and dispose of that aliquot after the fourth use/thaw. After creating your mix, incubate for one hour at room temperature.

3. Centrifuge at max speed (~14k rpm) for 15 minutes and aliquot 5 μ L of mix into three other labeled tubes. Store the three aliquots in -20°C and use one tube to inject immediately. To use the other aliquots, allow to thaw at room temperature for five minutes and proceed to the microinjection protocol as normal.

Screen for edits

1. After injection, recover injected worms (P0s) to individual NGMA plates and place them in an incubator at 20°C for 3-4 days until most offspring (F1s) of the injected worm are a mix of young adults and L4s.
2. Screen plates for Dpy and Rol phenotype F1 worms and set aside any plates with either. Plates with many Dpy and Rol worms should be prioritized in the following step.
3. Single F1s from these plates for up to twenty worms per P0 plate. Singling F1s with the Rol phenotype will have the best chance of also having your edit but Dpy worms are also worth screening. Wild-type F1s on plates with many Dpy and Rol worms also can be screened, but these worms will be less likely to have the edit than the Dpy and Rol worms from the same plate. Note on each F1 plate from which P0 plate they came because you will want two independent edits derived from different P0s.
4. Allow the singled F1 worms to lay eggs for 1-3 days before single-worm lysis and screening, either by detecting band size change for deletion or by sequencing if screening for homologous repair edits. If detecting HR edits, be sure to check the chromatography reads as any potential edits are likely to be heterozygous for your edit.
5. Single 10 F2s from potential edits to homozygous and confirm the edit with a preference for wild-type (for *dpy-10*), then Rol, then Dpy worms if necessary. Sequence to confirm changes. Repeat singling (from the most recently screened plate with the desired edit) eight offspring. Genotype all offspring for two generations where all eight screened offspring from a plate are homozygous for the edit. These steps are necessary to confirm homozygosity before freezing/phenotyping. A cross to remove the *dpy-10* co-CRISPR edit might be required if your strain has a Dpy phenotype.