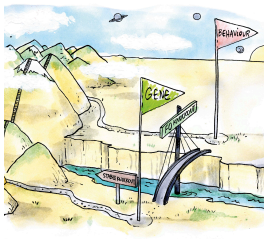
Nov 12, 2020 Version 1

F0 knockout—single gene V.1



DOI
dx.doi.org/10.17504/protocols.io.bfgyjjxw

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Protocol Citation: Francois Kroll 2020. F0 knockout—single gene. **protocols.io** **https://dx.doi.org/10.17504/protocols.io.bfgyjjxw**

Protocol status: Working

Created: April 23, 2020

Last Modified: November 12, 2020

Protocol Integer ID: 36088

Abstract

Remember to cite the publication if you use this protocol!

Get in touch for questions
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Protocol materials

- tracrRNA **Integrated DNA Technologies, Inc. (IDT)** **Catalog #**1072532
- Duplex Buffer **Integrated DNA Technologies, Inc. (IDT)** **Catalog #**11-01-03-01
- crRNA **Integrated DNA Technologies, Inc. (IDT)** **Catalog #**Custom Order
- Alt-R® S.p. HiFi Cas9 Nuclease V3 **IDT** **Catalog #**1081060

1 **crRNA selection**

This protocol is to disrupt one gene at three target sites.

We ordered all components from Integrated DNA Technologies (IDT).

For the published work, we used the predesigned crRNAs from IDT (https://eu.idtdna.com/site/order/designtool/index/CRISPR_PREDESIGN).

Wherever possible, crRNAs targeted three distinct exons while proceeding down the list from the best predicted crRNA. The sequences of all the crRNAs we have used can be found in Supplementary File 1.

We have made some suggestions with regards to which exons in the gene can be prioritised. They can be found in Discussion (section *Design of F0 knockout screens*) and Author response (question 7). In summary, we think an ideal set of three crRNAs target three distinct, asymmetrical (of length not a multiple of 3) exons, while avoiding exon 1.

2 **Re-suspend**

Do everything On ice

First, spin down the vials to make sure the pellets are at the bottom.

For 2 nmol crRNA: resuspend in 10 µL of Duplex buffer (each), i.e. [M] 200 micromolar (µM) stock.
For 5 nmol tracrRNA: resuspend in 25 µL of Duplex buffer, i.e. [M] 200 micromolar (µM) stock.

Vortex and spin down a couple of times.

Once resuspended, I would recommend aliquoting directly both the crRNA and tracrRNA, as RNA might be sensitive to the thaw-freeze cycles. For instance, aliquot the crRNA in ~ 4 µL aliquots and the tracrRNA in ~ 10 µL so each aliquot is used a maximum of three times. Keep track of how many times you have used each aliquot with marks on the Eppendorfs.

Store crRNAs and tracrRNAs at -80 °C

crRNA **Integrated DNA Technologies, Inc. (IDT)** **Catalog #**Custom Order

tracrRNA **Integrated DNA Technologies, Inc. (IDT)** **Catalog #**1072532

Duplex Buffer **Integrated DNA Technologies, Inc. (IDT)** **Catalog #**11-01-03-01

3 **Annealing**

In small PCR tubes;

- 1 µL crRNA [M] 200 micromolar (µM)
- 1 µL tracrRNA [M] 200 micromolar (µM)
- 1.51 µL Duplex buffer

Total 3.51 µL

One for each crRNA; typically 3x.

Place in a thermocycler: 95 °C for 00:05:00

crRNA annealed to tracrRNA is sgRNA; now at [M] 57 micromolar (µM)

4 **Dilute Cas9**

If bought from IDT, Cas9 is shipped [M] 10 µg/µL = [M] 61 micromolar (µM) .

Easiest is to dilute some for future experiments and keep it -20 °C .

For example:

- 9.6 µL Cas9
- 0.4 µL Cas9 buffer

Total 10 µL Cas9 [M] 57 micromolar (µM) .

Cas9 buffer (Wu et al., 2018. *Developmental Cell*):

- [M] 20 millimolar (mM) Tris-HCl
- [M] 600 millimolar (mM) KCl
- [M] 20 % volume glycerol

Alt-R® S.p. HiFi Cas9 Nuclease V3 **IDT Catalog #**1081060

5 Assemble RNP

In each PCR tube;

- 1 µL sgRNA
- 1 µL Cas9 [M] 57 micromolar (µM)

Total 2 µL

One for each crRNA; typically 3x.

Place in a thermocycler: 37 °C for 00:05:00

sgRNA assembled with Cas9 is RNP; now at [M] 28.5 micromolar (µM)

6 Pool RNPs

Pool 2 µL (or everything you can get) of each RNP from previous step.

Total ~ 6 µL if 3x.

Total RNP concentration is [M] 28.5 micromolar (µM) ; each RNP is at [M] 9.5 micromolar (µM) if 3x.

According to IDT (https://eu.idtdna.com/pages/education/decoded/article/genome-editing-how-stable-is-my-crispr-rna-cas9-rnp-complex), you can keep the RNPs at 4 °C or -20 °C or -80 °C for at least 10 weeks without loss of activity. You

can also read more here:
https://eu.idtdna.com/pages/education/decoded/article/stability-of-crispr-reagents-under-different-freezing-storage-and-thawing-conditions

I typically prepared the RNPs the evening before injections then stored the pool at 4 °C or -20 °C overnight before injection the next morning.

The RNPs may be sensitive to freeze-thaw cycles so re-using them might not recommended. Aliquoting the solution, eg. 2 µL in 3 small PCR tubes, and storing them at -20 °C or -80 °C could work.

7 **Injection**

Inject 1 nl in the yolk at the single-cell stage, before the cell inflates.

You are injecting:

- 28.5 fmol = 4700 pg Cas9
- 28.5 fmol = 1000 pg sgRNA
- If 3x; each sgRNA is 9.5 fmol = 333 pg

In my experience, the solution left a black stain in the yolk after a few seconds when injected successfully, so I did not use a dye.

If using multiple needles (for instance when injecting scrambled RNPs for control), try leaving the needle you are not using on ice on the side.

FREQUENTLY ASKED QUESTIONS

8 ***1- Can you confirm you inject in the yolk? Is it not better to inject in the cell?***

Yes, we have always injected in the yolk. We simply aim to inject in the centre of the yolk. Wu et al., 2018 (**10.1016/j.devcel.2018.06.003**) looked at yolk vs cell injections in great detail. Among others, they found that the RNPs are rapidly transferred to the cell. It is possible that injecting directly in the cell would create slightly more mutations. However, mutagenesis is not a limiting factor in our experience. We would actually be slightly more worried about generating *too many* double-strand breaks, especially when targeting multiple genes at once. Most importantly, yolk injections are much quicker, which means 1) all the eggs are injected rapidly just after they are laid; 2) higher Ns can be achieved,

which is especially useful for experiments where a population of F0 knockouts need to be phenotyped.

2- My gene has a single/two exons, how should I proceed?

We would simply place all three target sites on the same exon (or two on one exon). In the publication, this was the case for *mab21l2*. You can read more about this in the Author response (question 7). Try to spread out the target sites as much as possible, especially avoiding overlap between each crRNA binding site/PAM sequence. This is to avoid as much as possible situations where one RNP mutates the binding site/PAM of another RNP.

3- Did any F0 knockout failed to replicate a stable mutant line phenotype?

At the time of writing this protocol, we have targeted 13 genes and replicated all expected phenotypes. All examples are presented in the publication.

4- In vitro transcribed sgRNA are more economic – is it necessary to use synthetic crRNA and tracrRNA?

Yes. It is highly likely that synthetic RNAs (i.e. not in vitro transcribed) are necessary for the success of the method. You can find an explanation and references in Author response (question 8).

5- It is more economic to use Cas9 mRNA – is it necessary to use Cas9 protein?

Yes. We think it is unlikely that the method would work as well with Cas9 mRNA. You can find an explanation and references in Author response (question 2).

6- How early should I inject?

We think it is crucial to inject *very* early, i.e. at the single-cell stage before the cell inflates. The cell should still be flat, wrapped around the yolk. I generally set everything up for injections (calibrate the needles, etc), then remove the divider of a few breeding boxes and wait for 7–8 minutes next to it, then collect the eggs, put the divider back, inject these eggs. All the eggs are generally injected within the first 20 minutes after they are laid. If for any reason some eggs have started inflating or dividing, I start over with the next clutch. This may be eased a little if the studied phenotype does not require maximum penetrance.