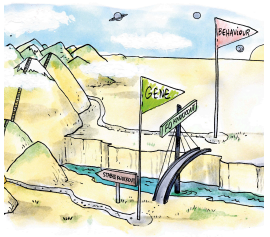
Apr 01, 2022 Version 3

F0 knockout—single gene V.3



DOI
dx.doi.org/10.17504/protocols.io.5qpvo52wdl4o/v3

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Protocol Citation: Francois Kroll, J Rihel 2022. F0 knockout—single gene. **protocols.io** **https://dx.doi.org/10.17504/protocols.io.5qpvo52wdl4o/v3**Version created by **Francois Kroll**

Protocol status: Working
W

Created: April 01, 2022

Last Modified: April 01, 2022

Protocol Integer ID: 60193

Keywords: single gene, gene

Abstract

Please cite **10.7554/eLife.59683** if you use this protocol.

Note; our **eLife publication** used **Version 2** of this protocol. Later version are subsequent improvements/simplifications.

Get in touch for questions/suggestions
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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

Troubleshooting

1 **crRNA selection**

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

To select your gRNAs, please refer to protocol

How to select the best gRNA(s) for frameshift knockouts in zebrafish
dx.doi.org/10.17504/protocols.io.81wgb6r5qlpk/v1

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
(vials of Duplex buffer are shipped with the tracrRNA, you do not need to buy this separately)

3 **Annealing**

In small PCR tubes;

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

Total 3.28 µL

One for each crRNA; typically 3x.

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

crRNA annealed to tracrRNA is gRNA; now at [M] 61 micromolar (µM)

4 **Assemble RNP**

In each PCR tube;

- 1 µL gRNA
- 1 µL Cas9 [M] 61 micromolar (µM)

Total 2 µL

One for each crRNA; typically 3x.

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

gRNA assembled with Cas9 is RNP; now at [M] 30.5 micromolar (µM)

5 **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] 30.5 micromolar (µM) ; each RNP is at [M] 10.1 micromolar (µM) if 3x.

According to IDT, 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**.

What I usually do is prepare the RNPs the afternoon/evening before injections and split the 6 µL of pooled RNPs in two aliquots of ~ 3 µL . I place one aliquot in the 4 °C fridge for injection the next day and place the other aliquot in the -80 °C freezer for future use if I need to repeat the experiment.

Once the RNPs are at -80 °C , I think you should worry more about freeze-thaw cycles than duration of storage. Accordingly, I throw any aliquot I use after injection just to be safe, even if there is a bit left. If you are experienced with injections, you could also split the 6 µL in three 2 µL aliquots that you keep a -80 °C , but I find that 2 µL is too low to reload the needle if I break it during injections, even if I load only ~ 0.9 µL .

6 Injection

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

If the cell has already inflated or undergone the first cell division, I would recommend skipping this clutch and starting again with fresh eggs.

You are injecting:

- 30.5 fmol = 5029 pg Cas9
- 30.5 fmol = 1070 pg gRNA
- If 3x; each gRNA is 10.1 fmol = 357 pg

The solution leaves a distinctive black stain in the yolk after a few seconds when injected successfully. Use this as a marker. Accordingly, I typically do not use a dye like phenol red in my injection mix.

FREQUENTLY ASKED QUESTIONS

7 ***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.