

Version 2 ▾

Mar 05, 2021

F0 knockout—single gene V.2

Francois Kroll¹, J Rihel¹¹University College London, University of London

1

Works for me

dx.doi.org/10.17504/protocols.io.bs2rngd6

Francois Kroll

SUBMIT TO PLOS ONE

ABSTRACT

Remember to cite the publication if you use this protocol!

Get in touch for questions

twitter – @francois_kroll

email – francois@kroll.be

DOI

dx.doi.org/10.17504/protocols.io.bs2rngd6

PROTOCOL CITATION

Francois Kroll, J Rihel 2021. F0 knockout—single gene. **protocols.io**

<https://dx.doi.org/10.17504/protocols.io.bs2rngd6>

Version created by [Francois Kroll](#)



WHAT'S NEW

Corrections: - previous version said Alt-R® S.p. HiFi Cas9 Nuclease V3 was used, but this was incorrect. Alt-R® S.p. Cas9 Nuclease V3 is correct. - previous version was referring to guide RNA as sgRNA but this was incorrect. It should be referred as gRNA, as it is not a single molecule but a crRNA:tracrRNA duplex.

LICENSE

————— This is an open access protocol distributed under the terms of the [Creative Commons Attribution License](#), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

CREATED

Mar 05, 2021

LAST MODIFIED

Mar 05, 2021

PROTOCOL INTEGER ID

47921

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. **100 Micromolar (µM)** stock.

For  **5 nmol** tracrRNA: resuspend in  **25 µl** of Duplex buffer, i.e. **100 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 Catalog #Custom Order

 **tracrRNA Integrated DNA**

Technologies Catalog #1072532




 **Duplex Buffer Integrated DNA**

Technologies 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 **100 Micromolar (µM)**
-  **1 µl** tracrRNA **100 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 gRNA; now at **57 Micromolar (µM)**

4 Dilute Cas9

If bought from IDT, Cas9 is shipped **10 µg/µL** = **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 **57 Micromolar (µM)** .

[Alt-R® S.p. Cas9 Nuclease V3 Integrated DNA](#)

Technologies Catalog #1081058

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

- **20 Milimolar (mM)** Tris-HCl
- **600 Milimolar (mM)** KCl
- **20 % volume** glycerol

[Alt-R® S.p. Cas9 Nuclease V3 Integrated DNA](#)

Technologies Catalog #1081058

5 Assemble RNP

In each PCR tube;

- **1 µl** gRNA
- **1 µl** Cas9 **57 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 **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 **28.5 Micromolar (µM)** ; each RNP is at **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 be recommended. Aliquoting the solution, eg. $2\ \mu\text{l}$ in 3 small PCR tubes, and storing them at -20°C or -80°C could work.

7 Injection

Inject $1\ \text{n}\text{l}$ in the yolk at the single-cell stage, before the cell inflates.

You are injecting:

- $28.5\ \text{fmol}$ = $4700\ \text{pg}$ Cas9
- $28.5\ \text{fmol}$ = $1000\ \text{pg}$ gRNA
- If 3x; each gRNA is $9.5\ \text{fmol}$ = $333\ \text{pg}$

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](https://doi.org/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.