Compiled CRISPR protocol
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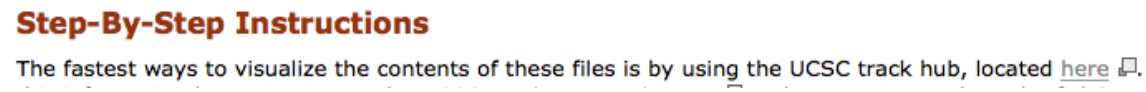
Outline:

I. Designing sgRNA
II. sgRNA production
III. Injection mix preparation
IV. Evaluating cutting efficiency
V. Tips and troubleshooting
    a. Removing 5' GG requirement

I. Designing sgRNA

A curated database of viable CRISPR target sites identified by the burgess lab is being maintained as a UCSC track. To access, click here: http://research.nhgri.nih.gov/manuscripts/Burgess/zebrafish/download.shtml

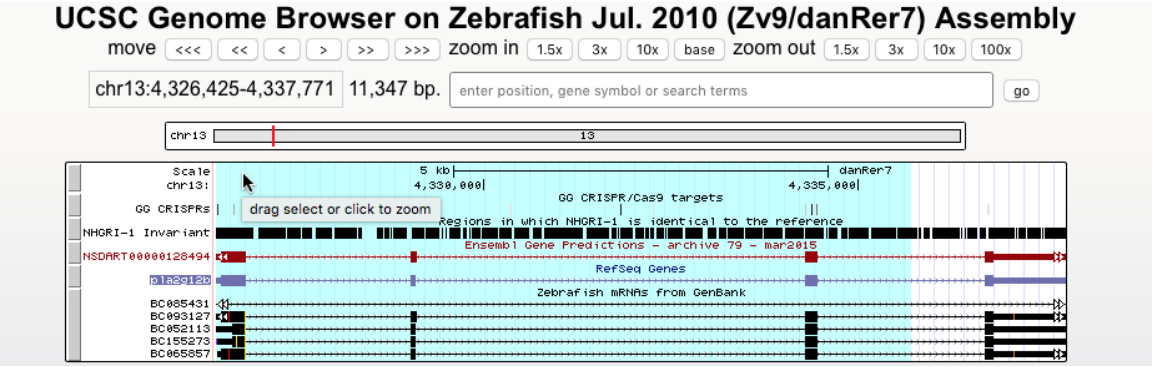
then scroll to the "Step by step instructions" and click on the "here" icon:

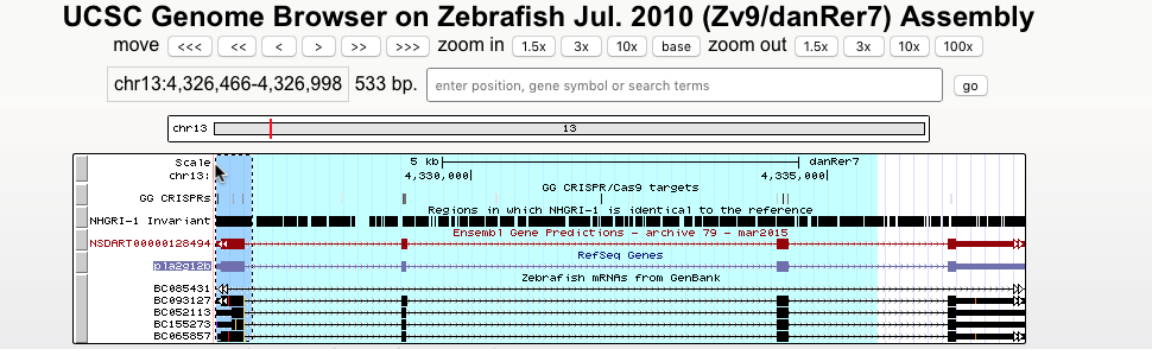


Use the search bar at the top of the page to identify your region of interest (ex. by searching "pla2g12b").

Set the following tracks to dense and hide all others:
-Zebrafish Genomics / GG Crisprs
-Zebrafish Genomics / NHRGI-1 Invariant
-Mapping and Sequencing / Base Position
-Genes and gene predictions / Ensembl genes
-mRNA and EST / Zebrafish mRNA

by placing your mouse in the scale/chromosome position region of the track display, you will be able to click and drag over a region of interest to highlight it, and you will be prompted to zoom in on that region. Use this to highlight a region of interest, for example from exon 1 (shown below) to try to identify CRISPRs that generate mutations early in the coding sequence that are likely to be mutagenic.





You should be zoomed in enough to see the GG CRISPR track, which will have either a ">>>>>>>>" or "<<<<<<<<" logo to indicate the direction of the target site. Click on whichever CRISPR you'd like to design first (should be early in the coding sequence and ideally invariant) and you will be taken to a page describing the feature.

Copy the "item" sequence from the top of the page which you can use to generate your CRISPR:

GGTGCAGGTTTTCCCTCAAC copied from:

Note that your target site will sometimes be on the forward strand and sometimes on the reverse, but copying this sequence will target the correct strand. In general

we will be using “GG” Crisprs, but you can relax the 5’ GG requirement if need be (see troubleshooting). Repeat this process for all viable targets (pla2g12b has 2 viable crisprs in each of the first two exons.

To generate a sgRNA targeting this site, simply add your target-specific sequence into the primer template below:

5′-TAATACGACTCACTATA-GGN($_{18\text{-}20}$)-GTTTTAGAGCTAGAAATAGC-3′

So the final primer sequence becomes:

5′-TAATACGACTCACTATA-GGTGCAGGTTTTCCCTCAAC-GTTTTAGAGCTAGAAATAGC-3′

The 5’ sequence added to the primer contains the T7 promoter, and the 3’ addition is simply complementary to the universal reverse primer sequence.

>SF-MS-73-CRISPRtail
AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTG
CTATTTCTAGCTCTAAAAC

By annealing the universal tail to the gene-specific forward primer and “filling-in” the oligo with polymerase, it is possible to generate sgRNAs without cloning.

II. sgRNA production

Once your oligos arrive, resuspend and dilute to get 10 uM working stock.

-Set up a PCR reaction using a high-fidelity polymerase (such as Phusion):

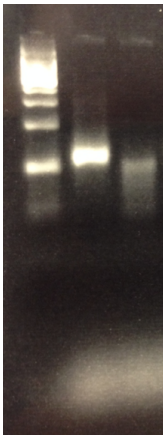
I used the Phusion polymerase from Thermo Scientific (#F-530S), and set up a 50 uL reaction:

H2O	33.5 uL
5x HF buffer	10 uL
10 mM dNTPs	1 uL
forward primer	2.5 uL
CRISPR tail primer	2.5 uL
Phusion polymerase	.5 uL

Note that the primers will be priming to each other and no template will be added. The oligos could theoretically be “filled in” with a single cycle, but since the polymerase is rate-limiting it may be beneficial to include more cycles. The amplicon will be about 123 bp, so a 15-30 second extension time is more than sufficient.

Cycle 1 (1x): 98C 30 s
Cycle 2 (15x):
-98C 10 s
-55C 30 s
-72C 30 s
Cycle 3 (1x): 15C hold

Below is a gel showing a "fill-in" reaction shown above, Lane 1 = 100 bp ladder, Lane 2 = reaction Lane 3 = negative control (no polymerase).



Once the reaction has completed, use a Qiagen PCR purification kit to purify the oligo. Elute in a very small volume of water (20 uL), and nanodrop.

The double-stranded sgRNA template now needs to be in-vitro transcribed to generate the sgRNA using the MxiScript T7 kit.

H2O	to 20 uL
Template (filled-in oligos)	1 ug (in <8 uL)
10x buffer	2 uL
75 mM ATP	2 uL
75 mM GTP	2 uL
75 mM CTP	2 uL
75 mM UTP	2 uL
T7 enzyme	2 uL

Incubate at 37C for at least 2 hours (ideally 4 hours or overnight)
-add 1 uL turbo DNase to reaction and incubate at 37C for 15 minutes
-Prepare an RNase-free 1.5 mL epitube of stop/precipitation solution by adding 15 uL Ammonium Acetate stop solution and 115 uL nuclease free water.

-Pipette the eniter reaction (20 uL) into the stop/precipitation solution and mix by pipetting
-Add 2 volumes (300 uL) ethanol and mix by inversion
-Chill at -20C for 30 minutes or greater
-Pellet the precipitated RNA by centrifuging at max speed for 30 minutes in the deli case centrifuge.
-Remove excess precipitation solution by decanting and pipetting with a p20 pipette
-Resuspend the RNA in nuclease free water. For the initial resuspension, I saw a large RNA pellet and used 40 uL of water to resuspend, giving a concentration of about 3000 ng/uL. An ideal stock concentration is about 1500 ng/uL, so be careful not to resuspend your RNA in too large of a volume or it will be too dilute. (less dilute sgRNA concentrations are still useful, it is not necessary to repeat unless concentration is below ~300 ng/uL.

-Use a 2 uL aliquot to nanodrop the RNA and then re-dilute and aliquot if necessary (~1500 ng per uL is ideal).
-Mix another 2 uL aliquot with 8 uL ambion loading buffer II and load onto a gel to verify that the RNA band is intact.

III. Injection mix preparation

Much higher cutting efficiency can be achieved by injecting CRISPR protein that is already complexed to sgRNA than could be achieved with mRNA injection. We have purchased Cas9 protein from NEB (EnGen Cas9, M0646T) that alleges to be at a concentration of 3.22 mg/uL, but my calculations indicate it is closer to 250 ug/uL. A good starting point for injections is 500 ng/uL Cas9 protein, complexed to 1:1 or 2:1 molar ratio of sgRNA to Cas9, which corresponds to about 330 ng/uL of sgRNA (for a 2:1 ratio, with sgRNA in excess). To prevent precipitation of the Cas9 in the injection mix, it should be buffered with KCl to a final concentration of 300 mM.

If the sgRNA has been stored at 1500 ng/uL, then all reaction components are 5x and the same volume of each can be added to the injection mix as shown below. Adding component in the order shown below ensures that the RNAse inhibitors in the phenol red will protect the sgRNA, and that the KCl prevents cas9 precipitation.

	Stock concentration	**Final concentration**	**Volume**
H20	NA	NA	.5 uL
5x Phenol red	2%	0.40%	.5 uL
5x KCl	1.5 M	300 mM	.5 uL
5x EnGen Cas9	2.5 ug/uL	500 ng/uL	.5 uL
5x sgRNA	1500 ng/ul	300 ng/uL	.5 uL
			2.5 uL total

1-2 nano-Liters of injection mix can then be injection into pre-1-cell stage embryos.

IV. Evaluating cutting efficiency

Cutting efficiency can be evaluated by PCR and subsequent polyacrylamide gel electrophoresis as described here:

Ota, Satoshi, and Atsuo Kawahara. "Detection of Multiple Genome Modifications Induced by the CRISPR/Cas9 System." Methods in molecular biology (Clifton, NJ) 1451 (2016): 53.

In essence, if cas9 introduces mutations, then the insertions/deletions will result in small changes in band length. Also, heteroduplexes of short DNA fragments migrte much slower than their perfectly complementary counterparts, providing a stereotypic band pattern.

Design primers that amplify a 100-300 bp product centered around your target site (or sites).

Following injection, extract DNA from injected fish by adding the fish to 100 uL of 50 mM NaOH and heating to 95C for 15 minutes (or use a tail biopsy, and boil in 20 uL NaOH for 10 minutes). Following heating neutralize with $1/10^{th}$ of a volume of 1 M Tris-HCl pH 8.1 and use in PCR reaction.

Pour a 10% polyacrylamide gel using a 40% stock solution of 19:1 acrylamide:bis-Acrylamide

Volume per gel (mL):	8	ml
Number of gels:	4	
Total volume:	32	ml
Gel percentage:	10	

Water:	19.75	ml
40% Acrylimide/Bis	8	ml
1.5 M Tris pH 8.8	4	ml
10% APS	0.25	ml
TEMED	0.02	ml

Run the PCR product on the gel for 275 volt-hours (50 volts for 30 minutes, 125 volts for 2 hours).

Stain the gel with a dilute ethidium bromide solution and image.

V. Tips and troubleshooting

When targeted to pla2g12b, cutting efficiency with a combination of oligos targeted to exon 1 was sufficiently high to result in a “darkened yolk” phenotype in ~100% of the injected embryos, and the exon 2 oligos had a similar result but appeared to generate slightly clearer yolks, indicating an intermediate hypomorphic phenotype.