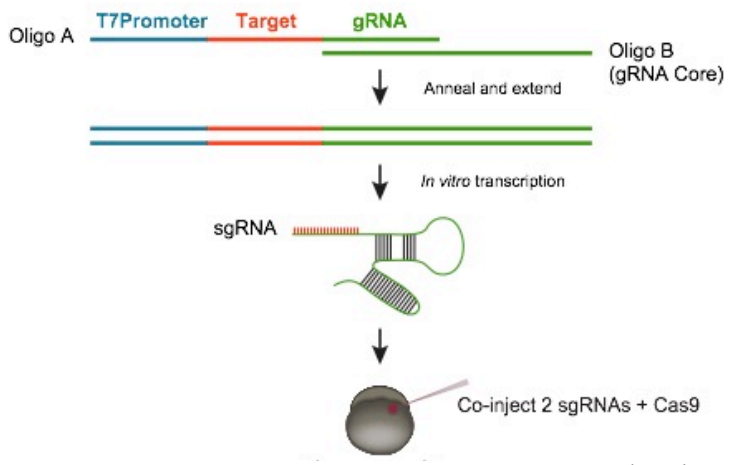
CRISPR/Cas9 Mutagenesis protocol for zebrafish

(Gaurav Varshney, Shawn Burgess Lab, NHGRI/NIH)

Last updated 9/2/2015

Cloning-free sgRNA synthesis:



Step1: Design 20-nt target sequence- CRISPR targets can be designed using various online tools (e.g. MIT CRISPR Tool, ChopChop, CasBlastr and many others).

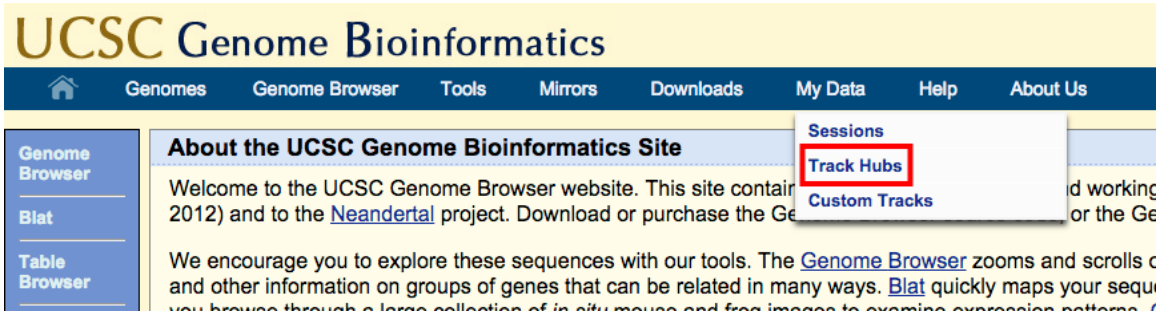
For the convenience of the zebrafish community, we computed all possible CRISPR targets in zebrafish genome, and these targets are available as custom tracks to be used with *UCSC genome browser* or *Ensembl genome browser.*

We recommend targets should either start with GG for T7 promoter, or GA for SP6 promoter, for efficient transcription. Targets with 1 or 2 mismatches at the 5′ will have negative impact on target activity.

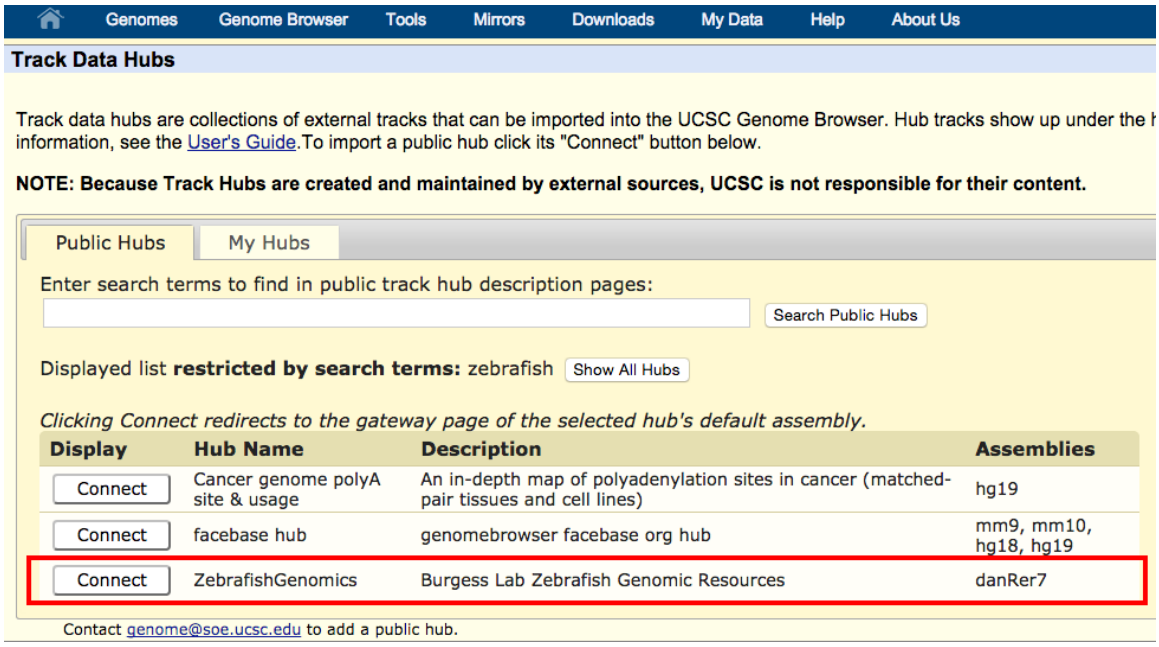
How to get CRISPR Target Sequence from UCSC Genome Browser:

Step 1.1

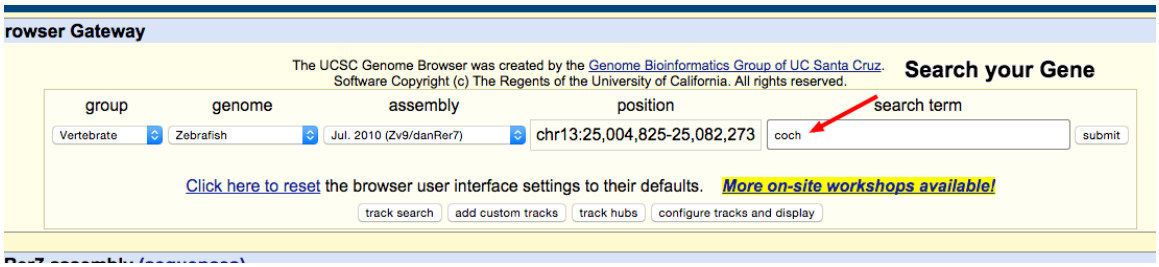
Go to http://genome.ucsc.edu and select track hubs from top menu



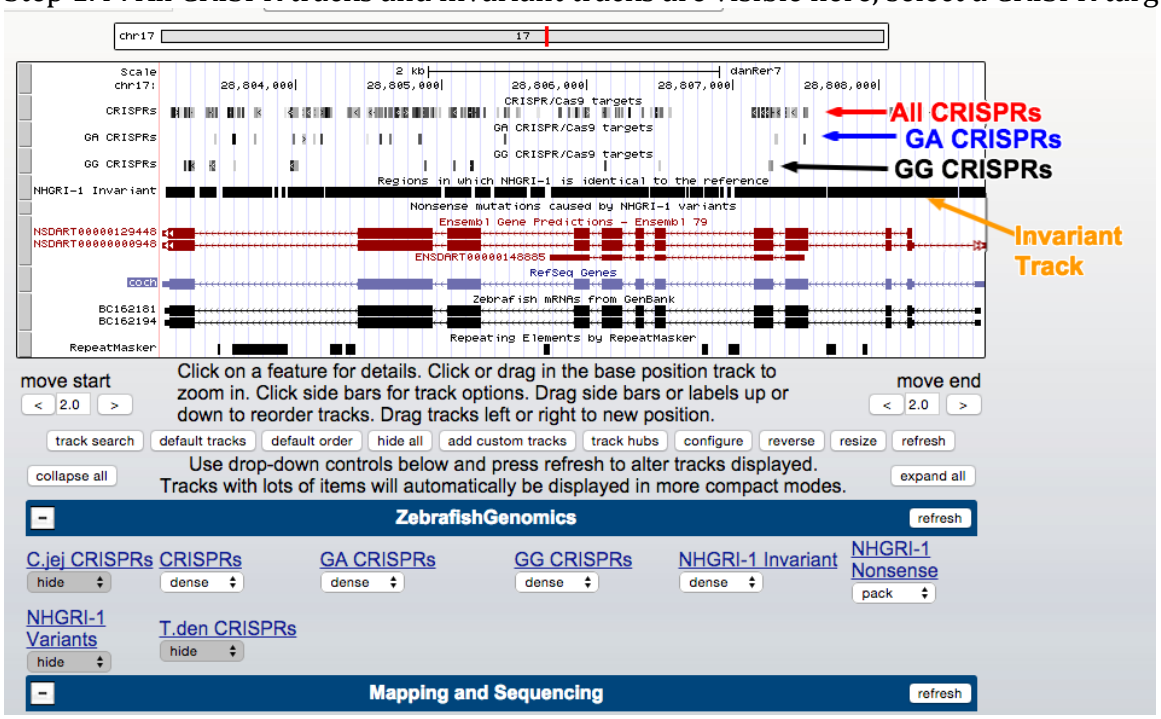
Step 1.2 Select Zebrafish Genomics and click connect, it will take you to zebrafish genome page.



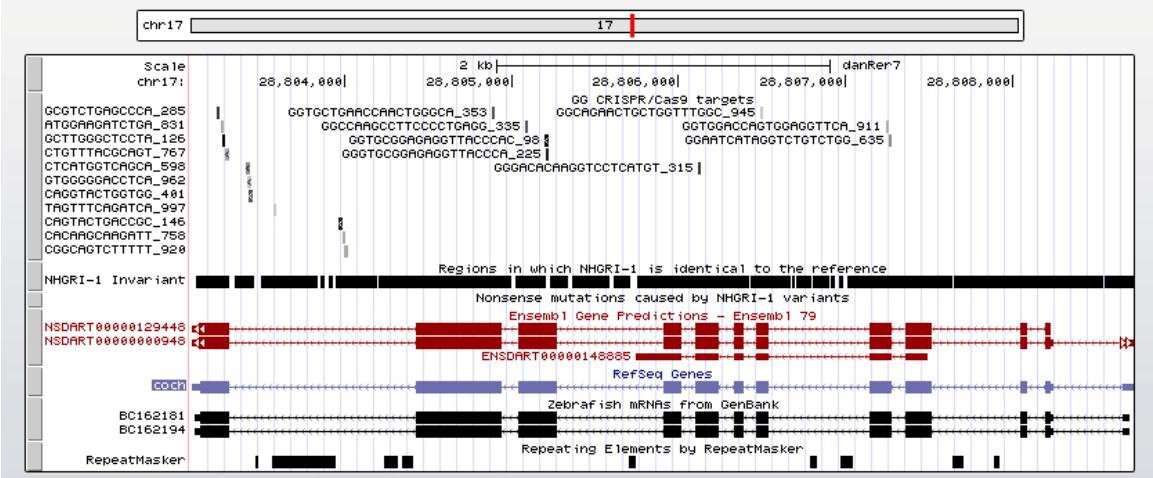
Step 1.3 Search for your favorite gene



Step 1.4 : All CRISPR tracks and invariant tracks are visible here, select a CRISPR target.



Step 1.5 Clicking on any CRISPR target will take you to this page, click on the target to get sequence



Step1.6 Copy CRISPR target sequence from here

GG CRISPR/Cas9 targets (GGGTGCGGAGAGGTTACCCA_225)

Item: GGGTGCGGAGAGGTTACCCA_225
Score: 841
Position: chr17:28805204-28805223
Genomic Size: 20
Strand: -
View DNA for this feature (danRer7/Zebrafish)

Go to GG CRISPRs track controls

Data last updated: 2014-11-07 05:26:46

Step 2: Add T7 promoter (GG) or SP6 promoter (GA) sequence at the 5' to target sequence, and 20-nt overlapping sequence at the 3'.

Target sequence(20nt): GGGTGCGGAGAGGTTACCCA
T7 Promoter (17nt): **TAATACGACTCACTATA**
OR
SP6 Promoter (17nt): ATTTAGGTGACACTATA

Overlapping crRNA-TracrRNA sequence (20nt): **GTTTTAGAGCTAGAAATAGC**

Step 1.7 Order *oligo1* as follows:
For T7
TAATACGACTCACTATAGGGTGCGGAGAGGTTACCCA**GTTTTAGAGCTAGAAATAGC**

Or
For SP6

ATTTAGGTGACACTATAGAAGGGCCCTGAGGTGGTGC**GTTTTAGAGCTAGAAATAGC**

Step 1.8 Order *oligo2* as follows (generic, order once):

5′- AAAAGCACCGACTCGGTGCCACTT TTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCT ATTTCTAGCTCTAAAAC -3′

Step 2 Oligo Assembly and Preparation of sgRNA

5X Buffer	5μl
10mM dNTP	0.5μl
Oligo1 (10μM)	1μl
Oligo2 (10μM)	1μl
Phusion Polymerase	0.5μl
Water (RNAse free)	17μl

Conditions:

1. Denature at 98°C for 2 minutes
2. Anneal at 50°C 10 minute
3. Extend at 72°C 10 minutes
4. Run **2.5% Agarose Gel**, you should see a **120 bp** band when 3-5μl loaded on gel

Step 3 In vitro Transcription using assembled oligo as template

Use 2-3 μl assembled oligos and proceeds for IVT reaction using **T7 quick High yield RNA synthesis kit (NEB)**

Reaction:

2X NTP mix	10μl
Assembled oligos	3μl
T7 polymerase	1μl
Water	6μl

Incubate at 37ºC for 2-16 hours

Add 1μl DNAse (RNAse free DNAse from the kit) and incubate at 37ºC for 15 minutes.

Precipitation/purification of guide-RNA

A. Purify RNA using isopropanol/sodium acetate

- Add 75 μl water to the reaction
- Add equal volume of isopropanol (100 μl)
- Add 1/10 volume of sodium acetate or ammonium acetate (pH5.2)
- Chill at -20ºC for ~30 minutes
- Precipitate RNA by spinning at maximum speed for 15 minute at 4ºC
- Wash pellet with 70% ethanol (200 μl)
- Spin at maximum speed for 5 minutes and dry pellet

- Dissolve pellet in 20 μl water

Or

B. Purify RNA using Zymo RNA Clean & Concentrator-5 **(# R1015)** kit as per manufacture's instruction

Check the quality of RNA; 1-2 μl on **2.5% gel agarose gel**

Nanodrop the RNA concentration, aliquot and store at -80°C

Step 4 Preparation of SpCas9 mRNA

1. Digest ~5-10μg **pT3TS-nCas9n** plasmid with Xba1 (plasmid addgene #46757 from ***Jao et al 2013***)
2. Purify digested DNA with QIAprep column (QIAGEN 27104)
3. Run on 1% gel.
4. Use ~500ng-1μg DNA as template for in vitro transcription reaction
5. Use mMESSAGE mMACHINE T3 kit Life Technologies (AM1348) and follow the instructions in the manual.
6. Precipitate RNA by LiCl followed by washing with 70% ethanol
7. Check the quality of RNA on 1% agarose gel (I use 1% agarose gel but denatured RNA gel can also be used) usually one band ~4.5 kb.
8. Quantify on nanodrop (>1μg/μl), aliquot and store at -80°C.

Embryo Injections:

Recommended amount:

Inject 25-50pg sgRNA (per target) and 300pg Cas9.

Note: To see phenotypes in injected embryos (biallelic) inject in cell (NOT YOLK)

You may use **CRISPR-CALC**, a microinjection calculator for calculating amounts of sgRNA and Cas9. (Download from CRISPRz website)

For **mutant Identification**, we use CRISPR-STAT or Sequencing. CRISPR-STAT protocol is on CRISPRz website.

Reference:

Carrington B, Varshney GK, Burgess SM, Sood R. **CRISPR-STAT: an easy and reliable PCR-based method to evaluate target-specific sgRNA activity.** Nucleic Acids Res. 2015 Aug 7 10.1093/nar/gkv802

Questions or comments, please email:
Shawn Burgess: burgess@mail.nih.gov
Gaurav Varshney: varshneygk@mail.nih.gov

If you use this protocol, please cite:

Varshney GK, Pei W, LaFave MC, Idol J, Xu L, Gallardo V, Carrington B, Bishop K, Jones M, Li M, Harper U, Huang SC, Prakash A, Chen W, Sood R, Ledin J, Burgess SM. High-throughput gene targeting and phenotyping in zebrafish using CRISPR/Cas9. Genome Res. 2015 dx.doi.org/10.1101/gr.186379.114

We are curating validated CRISPR Targets in a database, CRISPRz at http://research.nhgri.nih.gov/crisprz
If you wish to **add your data**, please contact us. CRISPRz will have the most updated protocols and tracks from the Burgess lab.