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ZF mutants and TG lines by NHEJ using CRISPR/Cas9

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A beginners guide to CRISPR

(outstanding scientists can skip this bit)

In short, to knock in a reporter sequence to a specific target site one needs to induce double-stranded breaks into both, a genomic region of interest and a plasmid containing the reporter gene in single-cell embryos. Non-homologous end joining will lead to DNA repair – in some cases the cut plasmid will be inserted into the cut genomic region during the repair. To that end one has to inject – *cas9* mRNA (for cleavage), single-guide RNA (sgRNA) against the genomic target sequence (to target Cas9 to a specific genomic site), a donor plasmid (with the reporter sequence) and another sgRNA against the donor plasmid (to target Cas9 to cleave the donor plasmid).

(For knock-outs, NHEJ can be used in the same way targeting a coding sequence but without injecting a donor plasmid and sgRNA targeting the donor plasmid).

A good introduction to the method and very nice protocols are provided by [1-5]. Talbot [4], Gagnon [3] and Auer [5] protocols combined are super detailed and clear. [6] is a paper about precise in-frame knock-ins using homology arms (not part of this protocol but just for reference).

Things to think about in advance (see further for details):

1. Decide whether to put the sequence under an endogenous promoter or use a minimal promoter driving the reporter. Second option gives higher expression levels and the insertion direction is irrelevant. Hsp70 promoter has been used, see [2].

2. Decide whether to target the reporter inside the gene (either under the endogenous promoter or with an added minimal promoter) or in the UTR (an enhancer trap with an added minimal promoter). In theory there are many options, in practice there might not be too many good sgRNA target sequences to choose from depending on the gene.

3. Design your bait-sequence and sgRNA pairs:

    a) Genomic bait sequence (target sequence where the reporter is inserted into) and sgRNA against it generated by PCR [3] (no need to clone it into a plasmid).
    b) Donor plasmid (containing the reporter to be knocked in) and sgRNA against a bait sequence it contains. You can use bait sequences that are known to work such as eGFP [5] (unless GFP is the reporter that is being knocked in of course), Mbait or Tbait [2].

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1. Target site selection

For knock-outs target constitutive exons (preferably 5'), conserved, functionally relevant, SNP-free regions. For knock-ins use minimal exogenous promoter and target the UTRs or introns to try avoid disrupting the endogenous gene [2] or knock-in under endogenous promoter using E2A linker for polycistronic expression [1]. For precise in-frame insertions use homology arms - not in this protocol, but see [6].

Online tools are available to choose the target site:

https://chopchop.rc.fas.harvard.edu/index.php
http://zifit.partners.org/ZiFiT/Disclaimer.aspx
http://crispr.mit.edu/

It's useful to compare all of them. For example CHOPCHOP - enter your target sequence and go to toggle advanced options:

- Under CRISPR select GG for 5' requirements – these are preferred starting nucleotides for T7 RNA polymerase. If no targets can be found with this restriction try NG or GN option for 5' and use either SP6 promoter or change N to G manually in your primer to still be able to use T7.
- PAM motif (protospacer adjacent motif) – NGG sequence directly after Cas9 cut site (in the target sequence but not included into sgRNA)

CHOPCHOP will suggest target sites ranking them according to location, GC %, off targets etc. Clicking on individual targets will give more information including suggested primers for HRMA.

Use Ensemble (or UCSC Genome Browser) genetic variation tool to check your target sequence for SNPs – avoid or if not possible, order sgRNAs for each variant.

If no targets can be found you can also try a search with altered PAM motifs for an engineered Cas9 – see [7] for details.

2. Designing sgRNAs

(for protocol to synthesise sgRNAs – see section 6)

A single-guide RNA (sgRNA) is synthesised from a DNA oligo that contains:

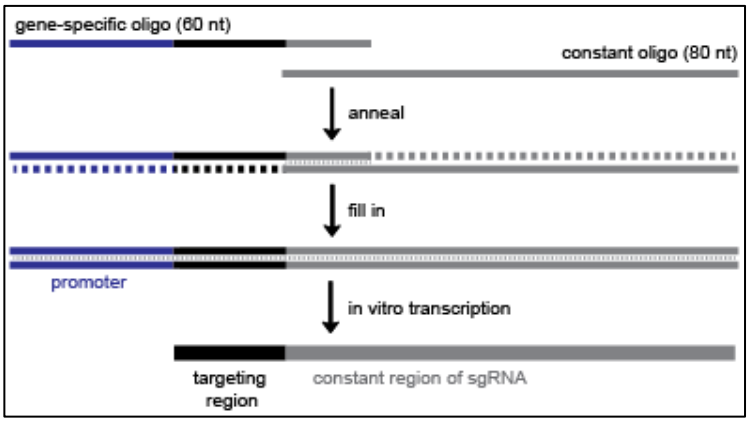
1. T7 promoter (for sgRNA synthesis)
2. *Targeting sequence* (to recognise genomic sequence that is targeted)
3. Constant sequence (Cas9 binding)
4. 5' GCG clamp can also be added (optional) (to stabilise the oligo)

GCGTAATACGACTCACTATA*GNNNNNNNNNNNNNNNNN*GTTTTAGAGCTAGAAATAGCAAGTT
AAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTT

Different options for generating sgRNA (see [4]supplementary for details on each):

1. Order sgRNA oligos as complete sequences – rather expensive
2. Plasmid-based method – if you want to have a nice storable sgRNA it can be cloned into a plasmid (see [4] for protocol). Suggested for sgRNAs that are likely to be used more often.
3. PCR-based short oligo method [3, 4] – suggested for target sequence sgRNAs (quick and cheap).

PCR-based sgRNA oligo design [3, 4]:



Basically, to avoid ordering the super-long oligo you break it into two, leaving overlapping complementary sequence to each, anneal them together and fill the other strand with T4 polymerase. Therefore, you need to order:

1. Gene-specific oligo with the 5' clamp, T7 promoter and *target sequence* (NB! Without the PAM, see section 1), and a sequence complementary to the constant oligo. The two Gs (*GG*) should be included in the target sequence (see section 1).

GCGTAATACGACTCACTATA*GG*NNNNNNNNNNNNNNNNN**GTTTTAGAGCTAGAAATAGCAAG**

2. Constant oligo – the constant sequence in all sgRNAs for Cas9 binding in reverse complement to anneal to the gene-specific oligo (the complementary bit is in bold):

AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAA**CTTGCTATT TCTAGCTCTAAAAC**

3. Designing donor plasmid and sgRNA

Look for existing plasmids from the literature before starting doing your own cloning. Things to consider – what is the donor sequence (GFP, Gal4, KalT4, GFPTx etc), what is the bait sequence (GFP, eGFP, Mbait, Tbait), are you inserting under an endogenous promoter or adding a minimal promoter, pA sequence. When inserting under the endogenous promoter the insertion has to be in frame and an E2A polylinker sequence has to be added before the inserted reporter (it will ensure that the reporter will be cut from the 5' endogenous sequence it is synthesised together with).

Donor plasmids we already have in plasmid stocks:

1. Gbait-hsp70Gal4 from [2] – to knock in gal4 under hsp70 promoter using eGFP bait sequence
    - Stock plasmid Gbait-hsp70Gal4 nr 1388
    - Kan resistant
    - Use together with sgRNAeGFP1 [5] (see sgRNA section)
2. eGFP-bait-E2A-KalT4-pA from [5] – to knock in KaltT4 under endogenous promoter using eGFP bait sequence
    - Stock plasmid eGFP-bait-E2A nr 1378
    - Kan and Amp resistant
    - Use together with sgRNAeGFP1 [5] (see sgRNA section)

Designing sgRNA for donor plasmid – see previous paragraph. sgRNAs we have (as a plasmid):

1. sgRNAeGFP1 from [5] – recognises eGFP bait sequence (can therefore be used for example to do a switch from eGFP line to KalT4 or to target donor plasmids with eGFP bait).
    - Stock plasmid gRNAeGFP1, nr 1379
    - Kan resistant
    - DraI linearisation, T7 transcription for mRNA

4. Designing and testing HRMA primers

CHOPCHOP recommends primers for each target sequence but check them in OligoAnalyser for self-dimerisation, hetero-dimerisation, melt-temperature etc. Ideally for Q-PCR the amplified sequence should be around 100-150bp (to detect changes in melt curves efficiently), melt-temperature for primers between 55-60°C.

Check the ordered primers by RT-PCR (for correct band-size on gel) and Q-PCR (for melt curve – aim for nice single peak!). For primer testing you can use either Biorad Supermix (as for HRMA, see section 8) or Promega GoTaq Sybr Green Master Mix (cheaper).

Q-PCR with Promega GoTaq Sybr Green mix:

Reaction mix (10ul)	Programme
5ul 2x Mastermix 1ul forward primer (10um) 1ul reverse primer (10um) 1ul of genomic DNA (HotShot, see section 8) 2ul of mQ	50°C 10min 95°C 5min 95°C 10sec X°C 30sec Plate read } 40x 95°C 10sec 65°C 5sec 65-95°C 5sec/step (0.5°C increments) + plate read

For HRMA to be accurate, you need to check your sequence for SNPs using Ensemble (or UCSC Genome Browser) (under genetic variation). If your amplified sequence contains SNPs, test your primers using Biorad Supermix on the WT embryos from parents whose progeny you plan to inject. Obviously best is to avoid sequences with SNPs if possible.

5. Cas9 mRNA synthesis

We use a plasmid from [8] – nls-zCas9-nls in pT3TS
- Stock CFCOcas9, nr 1380
- Amp resistant
- XbaI linearisation, T3 transcription for mRNA

1. Re-transform the plasmid,
- Plate on LB-Amp agar plates, grow 100ml midipreps
- Purify with Quiagen Plasmid Midi Kit and resuspend DNA in 100ul of nuclease free water.

2. Linearise the plasmid
- With XbaI
- Purify the reaction with PCR purification kit (Qiagen), elute with 20ul nuclease free water.

3. Transcription (Ambion mMESSAGE mMACHINE kit <u>for capped mRNA synthesis</u>)
- Work Rnase free!
- Keep the polymerase mix and 2x NTP/CAP on ice, but the buffer at RT
- Assemble reaction at RT in the correct order (buffer after water and NTP):

Reaction mix (20ul)	Programme
MQ up to 20ul 10ul 2x NTP/CAP 0.5 – 1ug template DNA 2ul 10x buffer 2ul RNA polymerase mix	Incubate at 37°C for 1-2h (2h especially when SP6 is used or transcript is <300bp)

- Remove DNA with TURBO DNase: add 1ul DNAase, incubate at 37°C for 15 minutes
- Store 1ul as a no-polyA control for gel

4. PolyA tailing (Ambion PolyA-tailing Kit)

- Before adding the E-PAP enzyme to the reaction, store 1ul as a no-enzyme control for gel.
- Assemble the reaction in the correct order:

Reaction mix (100ul)	Programme
20ul Dnase treated transcription reaction 36ul Nuclease free water 20ul 5x E-PAP buffer 10ul 25mM $MnCl_2$ 10ul 10 mM ATP 4ul E-PAP	Incubate at 37°C for 1h Place on ice or store at -20°C

- Run an 1% agarose gel – before polyA-tailing control, before E-PAP control and E-PAP polyA.
- Cas9 should be around 1.5-2kb. PolyA tail should add about 150bp or more compared to control.
- Before loading to the gel, add the loading dye (containing 20mM EDTA or add EDTA to prevent RNA degradation caused by divalent cations from the tailing reaction) and heat the samples at 80°C for 10 minutes.

5. Purification (ammonium acetate/ethanol to remove unincorporated nucleotides)

- Add 50ul of 5M ammonium acetate (in the Ambion kit) and 200ul of 96% EtOH
- Incubate at -80°C until frozen (about 20min) (or at -20°C O/N for higher yields)
- Spin at max speed for 30min at 4°C
- Remove supernatant, wash with 1ml of 70% EtOH
- Spin at max speed for 5min at 4°C
- Remove supernatant, dry at RT until all EtOH has evaporated
- Resuspend in 50ul (or 30 if the pellet looks small)

Run on a gel, measure the concentration with Qubit and store in small aliquots at -80°C.
Before using with new sgRNAs test cas9 mRNA by injecting it with an sgRNA that is known to work.

6. Generating sgRNAs (PCR method) [3, 4]

Design constant and gene specific oligos (see section 2).

1. Anneal constant and gene specific oligo:

Reaction mix (10ul)	Programme
1ul gene specific oligo (100um) 1ul constant oligo (100um) 8ul mQ	95°C 5min 95°C –> 85°C -2°C/sec 85°C –> 25°C -0.1°C/sec 4°C hold

2. Synthesise the second strand with T4 polymerase (NEB)

Reaction mix (20ul)	Programme
10ul annealing reaction 2.5ul dNTP (10mM) 2ul NEB buf 2 0.2ul 100xBSA 0.5ul NEB T4 DNA polymerase 4.8ul mQ	Incubate at 12°C for 20min.

Purify the reaction with PCR purification kit (Qiagen). Elute in 30ul, run 1ul on gel (product about 120bp) and measure the concentration (about 100-300ng/ul)

3. RNA synthesis with HiScribe T7 High-Yield RNA Synthesis Kit (NEB) (<u>non-capped RNA synthesis</u>)

Work RNase free!
Assemble the reaction at RT in the following order:

Reaction mix (20ul)	Programme
Xul nuclease free mQ 2ul 10x buffer 2ul ATP (100mM) 2ul GTP (100mM) 2ul CTP (100mM) 2ul UTP (100mM) 1ug template 2ul T7 polymerase	Incubate at 37°C for minimum of 2h (3-4h seems to be sufficient). (For higher yield you can increase template amount to 2ug and incubation time up to O/N).

(Alternatively you can also use Ambion MEGAscript T7 Kit. If you have an SP6 promoter, there is no HiScribe Kit, so use Ambion MEGAscript SP6 Kit).

4. RNA cleanup

- Use Qiagen RNeasy Plus Mini Kit (or do a usual ammonium acetate precipitation as for cas9 mRNA – see section 5). Elute in 50ul.
- Measure the concentration with Qubit fluorometer rather than Nanodrop (Nanodrop gives a massive overestimation). The concentration should be in the range between 100-900ng/ul.
- Run 1ul on a freshly made gel (clean all equipment beforehand). In order to be able to estimate the size of the band, heat the RNA at 80°C for 5min before loading to gel.

7. Generating a donor plasmid

- Clone your own or use an existing plasmid (see section 3).
- Re-transform the plasmid, plate on LB-antibiotics agar plates, grow 100ml midipreps, purify with Quiagen Plasmid Midi Kit and resuspend DNA in 100ul of nuclease free water.
- No need to linearise the plasmid. Measure concentration with Qubit (dsDNA)
- **Store at 4°C, use within two (three) weeks**

8. Injection of sgRNAs, Cas9 and donor plasmid

The suggested concentrations of sgRNAs and *cas9* mRNA varies depending on the protocol. Each new *cas9* and sgRNA should be tested separately with a working sgRNA/*cas9*, respectively.

Concentrations should be measured with Qubit.

Minimum amounts to start with (per embryo)	Worked for me (per embryo, 1nl injections)
100pg sgRNA (each) 100pg of donor plasmid 300pg Cas9 mRNA	110-140pg 250pg 170pg

- Inject 1-2nl into the cell (if you have a donor plasmid) of early 1-cell embryo.
- Keep uninjected controls to check death rate and have a comparison for HRMA (for knock-outs)
- Check the injected embryos for expected fluorescent signal – expression is mosaic and often there is ectopic expression in the muscle (especially at earlier stages, possibly directly from the donor plasmid not from genomic insertion). Use PCR to confirm insertion if visual screening is not reliable.

9a. HRM analysis for sgRNA mutagenesis efficiency (for knock-outs)

HotShot preparation of genomic DNA from single embryos

- At 24-30hpf put 5 uninjected control embryos and 10 injected embryos into 96 well plate (one embryo per well).
- Make fresh 1x base and neutralisation solutions every time
- Add 25ul 1x base solution into each well
- Seal the plate and heat in the PCR machine at 95°C for 30min, cool to RT
- Add 25ul of 1x neutralisation buffer
- DNA is in the solution on top of tissue, store in the plate as it is at -20°C

Base solution (50x) -14.03g KOH crystals (1.25M final), 4ml 0.5M EDTA (10mM final), H_2O to 200mL
Neutralisation solution (50x) - 63.04g TRIS-HCl (2M final), H_2O to 200mL

HRMA with Biorad Precision Melt Supermix

Reaction mix (10ul)	Programme
5ul 2x supermix 0.5 ul forward primer (10um) 0.5 ul reverse primer (10um) 1ul of genomic DNA 3ul of mQ	95°C 2min 95°C 10sec X°C 30sec 72°C 30sec plate read } 40x 95°C 30sec 60°C 1min 65-95°C 10sec/step (0.2°C increments) + plate read

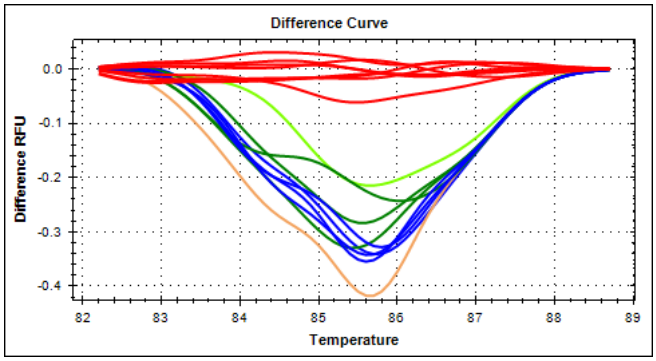
HRMA analysis

Strongly recommend reading this bit of the Talbot *et al* protocol [4].

- Open BioRad Precision Melt analysis – generate new melt file from file created from run
- Select wells containing WT embryos – check that the melt peaks and melt curves are all the same and clustered together. If some are not good or haven't amplifyed well, exclude those. If there are several clusters go back to checking SNPs and primer pairs (see section 4).
- Go to normalised view (melt curves where normalisation borders are placed before and after peak melting temperature to allow melt curves to be analysed independently from amplification levels) and adjust upper and lower temperature limit if necessary (according to melt peak temperature).
- With the WT embryo wells still selected, select wells containing injected embryo and set the WT cluster as the 'reference' cluster.

- If your sgRNA/Cas9 has introduced mutations there will be a shift in melt peaks of the injected embryos in comparison to the wild types. This will be most evident on the difference curve, where reference group (wt) is clustered togetherand set as RFU = 0. Most mutants will generate downward deflection as heteroduplex DNA usually melts at lower temperatures than homoduplex. Usually the deflection is around 0.2-0.5 difference-RFU in F0 (much less than that = low efficiency, less likely to get germline transmission). Different mutations generate different melt curves (different deflections) and therefore can be distinguished (do not cluster together).

Example:



- Red – uninjected controls clustering together
- Green/blue/orange – injected embryos showing a variety of melt deflections

- Together with melt analysis always check the amplification curves! (e.g. – additional melt peak at lower temperatures is likely to be primer dimers – shouldn't happen if the primers have been tested properly. A melt peak with a "shoulder" is a good indicator of a mutation causing melt shift etc).

Depending on the sgRNA efficiency, put x number of injected embryos into nursery.

9b. Illumina MiSeq (from Ida)

In addition/instead of HRM analysis one can use Illumina MiSeq to check the cutting rate of one's CRISPR.

Primer design

1. Design primers to amplify 160-180bp around the target site – make sure the target site is in the middle so that reads are obtained in both the forward and reverse direction
2. Append the following 5' tags to your forward and reverse primers respectively:

   Universal Tag F:
   5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-3'

   Universal Tag R:
   5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-3'

3. Test your primers on gDNA
   a. Usually a temperature gradient 55-65°C is required as these primers are very long and so optimisation can be tricky – try to ensure single band
   b. Final amplicon size should be approx. 230bp

Protocol

1. At 24hpf inspect embryos for death/ monster rate – note down for optimisation of injections.
2. For each injection, place ten embryos in one well of PCR tube/ plate (remove excess water)
3. HotShot DNA prep (see section 9a)
4. Use 1ul gDNA prep to amplify your target sequence using MiSeq primers. Make sure to use Platinum Taq High Fidelity so that mismatches are not incorporated as this will be detected in the results.

5. Run 1-2ul of PCR on 2% agarose
    a. If product is clean, use PCR purification kit to remove excess nucleotides and primers
    b. If not a single band, run entire PCR on a gel and cut out band at 220-230bp and gel purify
    c.
6. Dilute your amplicon to 15-25ng/ul in 10ul, ready to hand over to the person who is doing the subsequent processing.

MiSeq data processing sheet:

In order for the sequencing results to be analysed, extra information is required, so prepare a table with the following information:

ID	Guide RNA used	Forward primer	Reverse primer	Strand (0 = fwd, 1 = rev)	Amplicon sequence (PAM in capitals, rest lowercase)
Dmist6	GACCTTATGAAATTCTGCTGAGG	CCTGATCTCCCTCACCGCTTTC	TGTAGCGTTATCATGGCAGGTTC	0	cctgatctccctcaccgctttctgcataacgcttttccagtctgggagtgctcagctgggagagtctaaagtgaacgccagaccttatgaaatcctgctgAGGcaaaatctgggtaaggaatttaatgtcgcacccgacaataaacctgtcaaggcctgtcccatctacaaaaggagaacctgccatgataacgctaca

10. HRM analysis and sequencing of F1/F2 mutant carriers (from Karin, Ida)

- Outcross adult F0 with WT and extract genomic DNA from single embryos (see section 9).
- Perform HRMA and/or Illumina MiSeq to find founders that transmit mutations in germline. Use MiSeq or sequencing to check for the type of mutation present in carriers.
- Put 1 or 2 tanks of F1 embryos (heterozygous) with a good mutation in the nursery. Fin clip adult F1 and perform HRMA. PCR and sequence those with abnormal melt curve. Analyse sequences using http://spark.rstudio.com/yostlab/PolyPeakParser/. Alternatively – do MiSeq.
- Outcross F1 harboring frame-shift mutation with Wild-type fish.
- Get KASP genotyping primers from LGC Genomics. Fin clip adult F2s (heterozygous with same mutation) and identify carriers using KASP.
- Incross adult F2 - 25% progeny will be homozygous.

References:

1. Auer, T.O., et al., *Highly efficient CRISPR/Cas9-mediated knock-in in zebrafish by homology-independent DNA repair.* Genome Res, 2014. **24**(1): p. 142-53.
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3. Gagnon, J.A., et al., *Efficient mutagenesis by Cas9 protein-mediated oligonucleotide insertion and large-scale assessment of single-guide RNAs.* PLoS One, 2014. **9**(5): p. e98186.
4. Talbot, J.C. and S.L. Amacher, *A streamlined CRISPR pipeline to reliably generate zebrafish frameshifting alleles.* Zebrafish, 2014. **11**(6): p. 583-5.
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