Modular Assembly to generate site-specific zinc finger nucleases (ZFN)
This protocol entails two separate PCR steps: first, to separately amplify each of the 3 individual fingers in the correct backbone finger position for each particular ZFN; second, an overlapping PCR step to place the 3 zinc finger proteins (ZFPs) into the correct single contiguous fragment. This 3-finger ZFP is then cloned in frame with a FokI nuclease variant in a pCS2-backbone plasmid. Protocols for mRNA synthesis and subsequent microinjection into zebrafish embryos are standard and not covered in detail here.

First round PCR of individual zinc finger proteins (ZFPs).
Each individual ZFP is PCR-amplified using the appropriate primer set (F1 forward and reverse for F1; F2 forward and reverse for F2, etc.). It is important to note that each ZFP in our plasmid set can be found in each of the three backbones in different plasmids. Also, some plasmids contain different fingers in all three positions. Therefore, take care to make sure that you use the correct primer set that corresponds to the correct finger positions in the correct template plasmid. Based on the row and column information from the ZFN database, use the indicated plasmids as templates with the corresponding ZFP primers. Note that the conditions are slightly different for ZFPs in backbone 1 compared to backbones 2 and 3.

PCR reaction conditions:

template plasmid	1 μl (20 ng)
10x HIFI buffer	5 μl
10x HIFI dNTPs	5 μl
50x Adv2 HIFI polymerase	1 μl
10 μM primer pair	2 μl
H20	to 50 μl

PCR cycle conditions (for MJ Research Tetrad):

ZFP F1
Step 1: 98° C for 2 minutes
Step 2: 94° C for 30 seconds
Step 3: 68° C for 30 seconds
Step 4: 72° C for 20 seconds
Goto Step 2, 14 times
4° C forever

ZFP F2 and F3
Step 1: 98° C for 2 minutes
Step 2: 94° C for 30 seconds
Step 3: 57° C for 30 seconds
Step 4: 72°C for 30 seconds
Goto Step 2, 19 times
4° C forever

Confirm that the PCR was successful by running 3-5 μl on an agarose gel. We usually run a 2 to 3% gel using Bio-Rad LoRes Ultra. We recommend using Orange G loading dye because it will run ahead of and not block out the 100 bp fragments. For successful PCRs, run as above (make sure the gel is TAE) and purify using the Qiagen Gel Extraction Kit. Elute fragments with 30μL of EB and quantify (We use a NanoDrop-1000 spectrophotometer). Fragments can be stored at -20° C indefinitely.
Note: On the gel, F2 should be larger than F1.

Overlapping PCR
The F1 reverse/F2 forward and F2 reverse/F3 forward primers have sufficient overlap to allow overlapping PCR to generate a single contiguous fragment containing all three fingers in a single reaction. Please note that the PCR consists of two separate amplifications: first without the external primers, in order to favor annealing between the fragments and extension of a single 3-finger ZFP fragment; second, addition of the flanking primers and amplification of the single fragment.

Reaction conditions

ZFP1	30 ng (1-3 μl)
ZFP2	30 ng (1-3 μl)
ZFP3	30 ng (1-3 μl)
10x HIFI buffer	5 μl
10x HIFI dNTPs	5 μl
50x Adv2 HIFI polymerase	1 μl
dH_20	to 48 μl

Overlapping program
Step 1: 94° C for 2 minutes
Step 2: 94° C for 30 seconds
Step 3: 55° C for 30 seconds
Step 4: 72° C for 30 seconds
Goto Step 2, 2 times; PAUSE

Add 2 μl 10 μM F1for/F3Rev primers

Step 6: 94° C for 15 seconds
Step 7: 68° C for 30 seconds
Goto Step 6, 24 times
4° C forever

Run 3.0 μL of the product onto a 2% agarose gel to see if the reaction worked properly (expected product size is 300bp). Gel purify the remaining as described above and elute in 30 μL of EB. Fragments can be stored at -20° C indefinitely.

Digestion and Ligation
At this point the 3-finger ZFP cassette is digested with Acc65I and BamHI and cloned in frame with the appropriate FokI nuclease variant. Since the ZFN acts as a heterodimer, make sure that the ZFPs specific to 5' and 3' target sequences are cloned into the corresponding vectors: if the 5'ZFP is cloned in frame with the DD FokI variant, the 3'ZFP needs to be cloned in frame with the RR variant. There are also EL and KK paired variants; these appear to have lower nuclease activity. We are currently investigating whether or not they may reduce off-target cutting, but at this time we recommend starting with the DD/RR nucleases.

Digest PCR product.

3-finger ZFP PCR product	30 μL
10x NEB buffer 3	5 μL
10x BSA	5 μL
Acc65I (10U/μL)	2 μL
BamHI (20U/μL)	1 μL
H_20	8 μL

incubate at 37°C, at least 2 hours; overnight is best.

Prepare the appropriate pCS-FokI vectors as you would any other vector for subcloning.

Gel purify the fragments according to standard protocols. The ZFP fragment is approximately 300 bp, while the pCS2-Fok vector backbone is 4.7 kb; if you are using the vectors containing a Kanamycin "drop-out" cassette you should also see a 1 kb fragment.

Use standard protocol for transformation. We perform butanol extraction, followed by electroporation into Top10 cells. Select for clones on LB/Amp plates (100 μg/ml). You can PCR screen clones using primers in pCS2, or an anchor in pCS2 with any of the ZFP primers. We routinely miniprep and sequence 2 or 3 clones.

Injections.
If using the DD and RR FokI variants, we recommend starting with 10 and 20 pg of mRNA encoding each paired ZFN per embryo. If using EL and KK variants, start with 50 pg. Ideally, you want to see about 60 percent of your embryos are normal, the others are monsters or dead.

Genotyping.
Pool 20 to 30 normal and monster embryos (separately) at ~24 hpf; use uninjected embryos as a control. Isolate genomic DNA (method of choice; we have used crude preps – 10 mM Tris/0.1% SDS/proteinaseK – as well as cleaner preps – Qiagen DNAEasy). Perform PCR using primer pairs indicated for a particular ZFN site in the ZFN database. Digest PCR product with restriction enzyme indicated in database for a particular spacer. Run on agarose gel (2-3% BioRad LoRes ultra/TAE). Successful introduction of lesions will result in a small proportion of the PCR fragment left undigested. You should only see this shift in embryos injected with the ZFN pair.

Reagents

Orange G loading dye
10 mM Tris-HCl (pH 7.6)
0.15% orange G
60% glycerol
60 mM EDTA.

Primer sequences

F1 forward: 5’-GCGATGGGTACCCGCCCATATGCTTGCCC
F1 reverse: 5’-CACTGGAAGGGCTTCTGGCCTGTGTGAATCCGGATGTG

F2 forward: 5’-CATCCGGATTCACACAGGCCAGAAGCCCTTCCAGTGTCGCATCTGC
F2 reverse: 5’-ATGTCGCATGCAAAAGGCTTCTCGCCTGTGTGGGTGCGGATGTG

F3 forward: 5’-CAGGCGAGAAGCCTTTTGCATGCGA
F3 reverse: 5’-GCGTAGGATCCACCTGTGTGGATCTTGGTGTG