CRISPR plasmids from Chen and Wente labs

We have synthesized a Cas9 coding sequence with nuclear localization signals (nls) at both its amino and carboxyl termini and codons optimized for zebrafish expression (nls-zCas9-nls). Injection of *in vitro* transcribed *nls-zCas9-nls* mRNA with a gene-specific chimeric guide RNA consistently resulted in high mutagenic efficiency in both somatic and germline cells. Injected fish often exhibit loss-of-function phenotype, indicating prevalent biallelic inactivation. The injected fish produces predominantly mutation-carrying progeny. In addition, multiple loci can be efficiently targeted simultaneously in the same fish.

Identify CRISPR targets: $GGN_{19}GG$ (see graph).

Design primers: Forward: $TAGGN_{18}$; Reverse: $AAACN_{18}$.

Primer Anneal: Anneal the two oligos (2 µl of 100 µM stock each in a 20 µl 1x NEB buffer solution) by incubating the mixture at 95°C for 5 min, ramping down to 50°C at 0.1°C/sec, incubating at 50°C for 10 min, and chilling to 4°C at normal ramp speed (1°C/sec).

Cloning (one-step digestion/ligation): Ligate the annealed oligos to the T7cas9sgRNA2 vector by mixing the following components: 1 µl of annealed oligos, 400 ng of T7cas9sgRNA2 vector, 1 µl of 10x NEBuffer 3, 1 µl of 10x T4 ligase Buffer, 0.5 µl of BsmBI, 0.3 µl of BglII, 0.3 µl of SalI, 0.5 µl of T4 DNA ligase, and water to a total of 10 µl. Perform digestion and ligation in a single step in a thermal cycler with the following program: 3 cycles of 20 min at 37°C/15 min at 16°C, followed by 10 min at 37°C, 15 min at 55°C, and 15 min at 80°C (optional).

Transformation: Use 2 µl of the ligation product and plate 10% of the transformants to a LB/Amp plate.

Identification of the correct clones: The cloning protocol usually results in >90% of colonies with the correct insert. We routinely sequence two colonies with M13F(-21) primer to confirm the clones.

In vitro transcription:

gRNA: For making gRNA, digest the plasmid with BamHI, treat it with Proteinase K (incubate the digested mix in 100 µg/ml of Proteinase K and 0.5% of SDS at 50°C for 20 min), and purify the linearized plasmid (e.g., QIAprep column). Set up in vitro transcription using the MEGAshortscript T7 kit (Ambion/Invitrogen) with 100-400 ng of purified linearized DNA following the manufacturer's instructions.

nls-zCas9-nls RNA: For making *nls-zCas9-nls* RNA, linearize the template DNA by NotI (pCS2-nls-zCas9-nls) or XbaI (pT3TS-nls-zCas9-nls) digestion and purify the template as described above for gRNA template. Capped *nls-zCas9-nls* RNA is synthesized using mMESSAGE mMACHINE SP6 or T3 kit (Invitrogen) and purified using RNeasy Mini kit (Qiagen) following the manufacturer's instructions.

RNA purification: We use *mir*Vana miRNA Isolation Kit (Ambion/Invitrogen) to purify the gRNA. gRNA can be further concentrated by ethanol precipitation if desired. Using unpurified gRNA is also possible but the survival rate will decrease.

Injection: We mix RNAs in 0.05% phenol red, 120 mM KCl, and 20 mM HEPES, pH 7.0 (150 ng/µl of *nls-zCas9-nls* RNA and 50-100 ng/µl of gRNA are a good starting point for the injection mix). Inject ~ 1 nl of the mix directly into the cell (not the yolk) of 1-cell-stage embryos. The mutagenesis efficiency is dose-dependent and likely target-dependent. The doses of Cas9/gRNA can be adjusted accordingly to obtain the desired efficiency.