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Protocol for Genome Editing via the RNA-guided Cas9 Nuclease in Zebrafish Embryos[1]

1. *In vitro* synthesis of capped *Cas9* mRNA

The full length of humanized *Cas9* cDNAs with double NLS were cloned into pXT7 vector (Amp-resistant) and linearized by XbaI (NEB; cat#R0145T). Capped *Cas9* mRNA was synthesized using mMESSAGE mMACHINE mRNA transcription synthesis kit (Ambion; cat#AM1344).

Then *Cas9* mRNA was purified using RNeasy Mini Kit (QIAGEN; cat#74106).

Detailed information is as follows.

In vitro mRNA transcription (Ambion mMESSAGE mMACHINE kit)

Amount Component

10 µL 2X NTP/CAP

2 µL 10X Reaction Buffer

1 µg linear template DNA

2 µL Enzyme Mix

to 20 µL Nuclease-free Water

Note: DO NOT add GTP .

RNA purification (QIAGEN RNeasy Mini kit)

Notes before starting

Add 4 volumes of ethanol (96–100%) to Buffer RPE for a working solution.

1.1 Adjust the sample to a volume of 100 µl with RNase-free water. Add 350 µl Buffer RLT, and mix well.

1.2 Add 250 µl ethanol (96–100%) to the diluted RNA, and mix well by pipetting. Do not centrifuge. Proceed immediately to step 3.

1.3 Transfer the sample (700 µl) to an RNeasy Mini spin column placed in a 2 ml collection tube (supplied). Close the lid. Centrifuge for 15 s at ≥8000 x *g*. Discard the flow-through. Optional: If performing optional on-column DNase digestion, follow steps 1–4 of "On-column DNase digestion" (above) after this step.

1.4 Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid. Centrifuge for 15 s at

≥8000 x *g* to wash the membrane. Discard the flow-through.

1.5 Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid. Centrifuge for 2 min at ≥8000 x *g* to wash the membrane.

Optional: Place the RNeasy spin column in a new 2 ml collection tube (supplied). Close the lid, and centrifuge at full speed for 1 min.

1.6 Place the RNeasy spin column in a new 1.5 ml collection tube (supplied). Add 30–50 µl RNase-free water directly to the spin column membrane. Close the lid, and centrifuge for 1 min at ≥8000 x *g* to elute the RNA.

1.7 If the expected RNA yield is >30 µg, repeat step 6 using another 30–50 µl of RNase-free water. Alternatively, use the eluate from step 6 (if high RNA concentration is required). Reuse the collection tube from step 6.

2. gRNA target site design

Please refer to the two websites below for detailed information.

http://zifit.partners.org/ZiFiT/

http://www.genome-engineering.org/crispr/?page_id=41

Tip: Selected target sites should be sequenced before carrying on experiments, especially for non-coding genes.

3. *In vitro* synthesis of gRNA

gRNA scaffold was cloned into pMD 19-T vector (TakaRa; cat#D102A) (Amp-resistant).

Double strand DNA for specific gRNA synthesis was PCR amplified (Transgen; cat#AS211) using primers below. After gel extraction, gRNA was synthesized using T7 RNA Polymerase (NEB; cat#M0251S). Then gRNA was digested by DNase I (NEB; cat#M0303S) for 30 min, and purified using TRIzol (Sigma; T9424) to a final volume of 5~10 µl.

4. Injection system (for 4µl)

1.8nl of *cas9* mRNA (300 ng/µl) and gRNA (20 ng/µl) were co-injected into 1- or 2-cell-stage wild type embryos.

Stock Volume Final Concentration:

Phenol red 0.4μl

$MgCl_2$ -- 10mM

Cas9 mRNA -- 300ng/μl

gRNA -- 20 ng/μl

Nuclease-free H_2O --

Total 4μl

Tip: (Optional but highly recommended) Prepare the injection mixture right before use.

5. Cas9/gRNA mutation screens of F0 embryos by Sanger sequence and SURVEYOR

Sanger sequencing: Genomic DNA of single zebrafish embryos at 50 hpf was extracted. Then genomic region surrounding the CRISPR target site for each gene was PCR amplified and cloned into the *pEASY*-Blunt Cloning vector (Transgen; cat#CB101) for sequencing, carrying out sequences of 20 to 30 clones per embryo. We also perform sequencing of PCR fragments for gaining preliminary information on indels, based on sequencing traces from multiple templates around the PAM sequence.

SURVERYOR: A total of about 200-400 ng PCR products (TakaRa; cat#DRR001A) were subjected to a re-annealing process to enable heteroduplex formation:

95ºC for 10min,

95ºC to 85ºC ramping at – 2ºC/s, 85ºC for 1 min,

85ºC to 75ºC ramping at – 0.3ºC/s, 75ºC for 1 min,

75ºC to 65ºC ramping at – 0.3ºC/s, 65ºC for 1 min,

65ºC to 55ºC ramping at – 0.3ºC/s, 55ºC for 1 min,

55ºC to 45ºC ramping at – 0.3ºC/s, 45ºC for 1 min,

45ºC to 35ºC ramping at – 0.3ºC/s, 35ºC for 1 min,

35ºC to 25ºC ramping at – 0.3ºC/s, 25ºC for 1 min.

After re-annealing, products were treated with $MgCl_2$, SURVEYOR nuclease and SURVEYOR enhancer S (Transgenomic; cat#706020) following the manufacturer's recommended protocol, and analyzed on 10% urea poly-acrylamide gels. Gels were stained with 0.5μg/ml EtBr in 1X TBE for 20 minutes, wash in water for 20min and imaged with a

gel imaging system (Tanon). Quantification was based on relative band intensities.

Tip: We recommended to use TakaRa ex Taq (cat#DRR001A) for SURVEYOR assay. Otherwise, you may test other PCR enzymes to get ideal results.

6. Heritable F1 germ-line transmission:

We are raising 50 to 100 F0 fish for each locus, and will perform either 1) F0 tail fin DNA genotyping and then apply those positive F0 for genotyping their F1 embryos; or 2) directly genotyping F1 embryos from F0 sibling crossing. F0 Tail fin DNA genotyping works well in our hands for predicting heritable F1 germline transmission on our Tol2-based transgenesis protocol (Similar genotyping of F0 mouse tail DNA is widely used for predicting germline transmission in the mouse field). As soon as we have data on germline transmission efficiency in the *etsrp*, *gata4* and *gata5* loci, we will share it with the community.

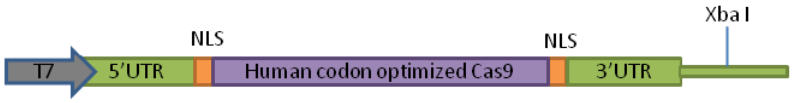

Fig 1. Schematic diagrams show the CRISPR system component Cas9. The full length of humanized *Cas9* cDNAs with double NLS were cloned into pXT7 vector (**Amp+**).

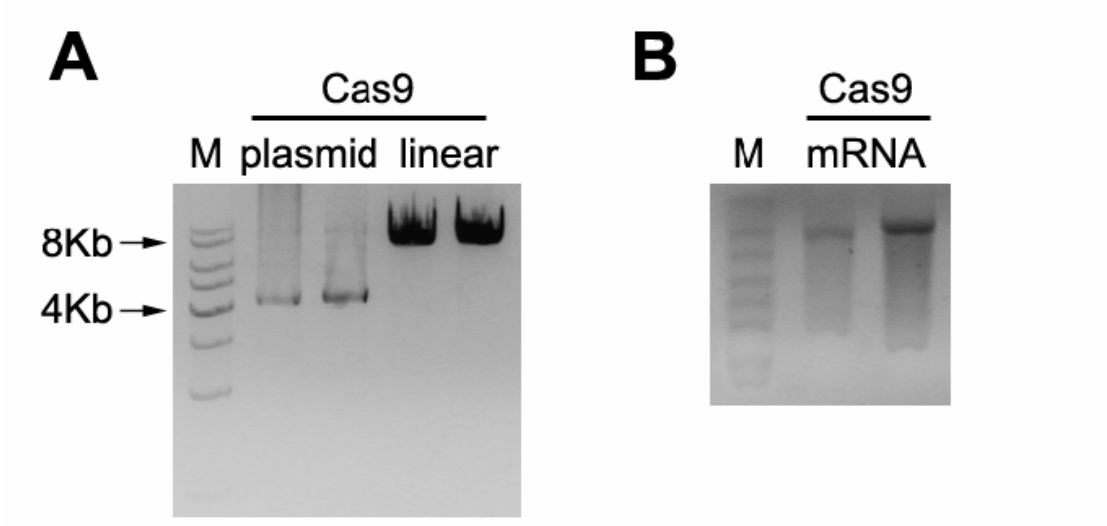

Fig 2. *In vitro* synthesis of capped *Cas9* mRNA. The pXT7-hcas9 plasmid was linearized by XbaI and synthesized by T7(Ambion)

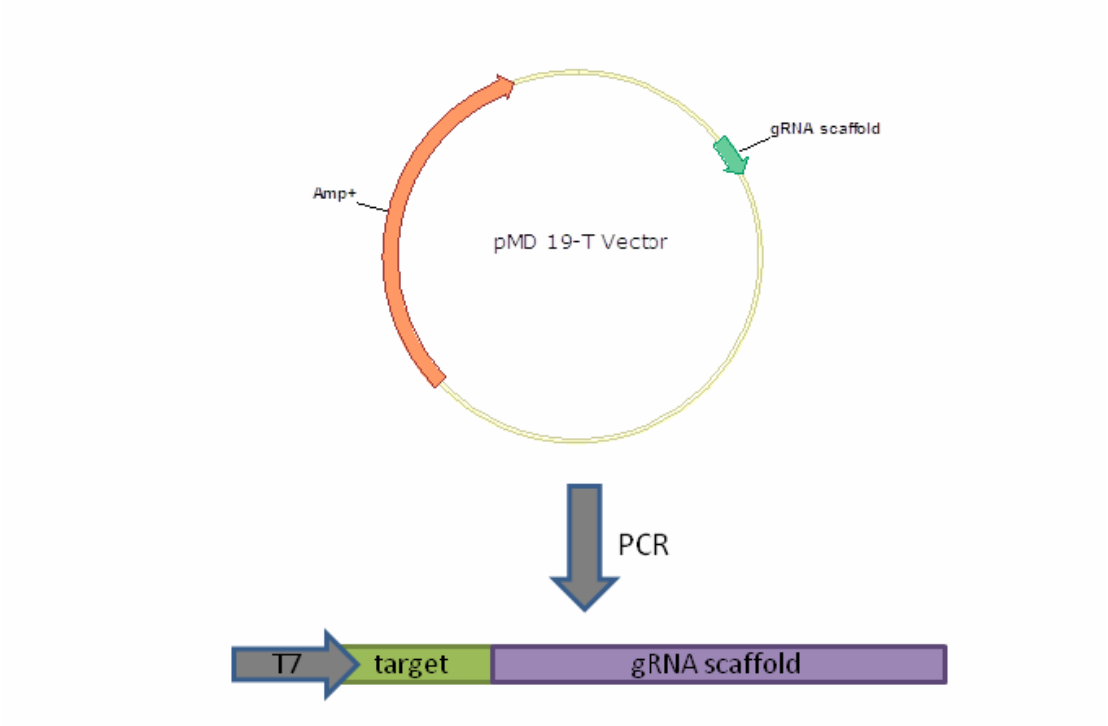

Fig 3. Schematic diagrams show the CRISPR system component gRNA. gRNA scaffold was cloned into pMD 19-T vector. Double strand DNA for specific gRNA synthesis was PCR amplified.

>PCR primers

Forward Primer: T7_19~23bp target sequence_20bp gRNA scaffold

Reverse Primer: 20bp gRNA scaffold

>gRNA scaffold sequence

GTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAA AAAGTGGCACCGAGTCGGTGCT

Reference

1. Nannan Chang, Changhong Sun, *et. al.* Genome editing with RNA-guided Cas9 nuclease in Zebrafish embryos. *Cell Research* (2013) 23:465-472.