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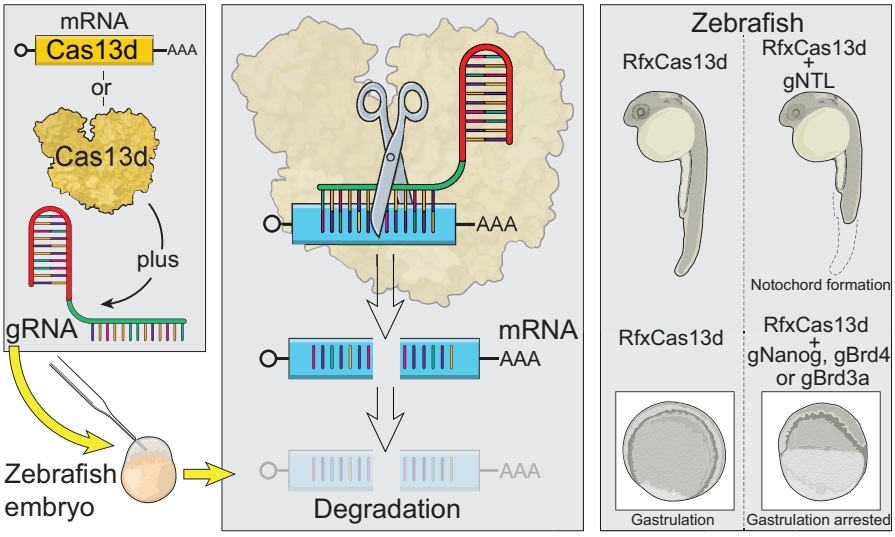
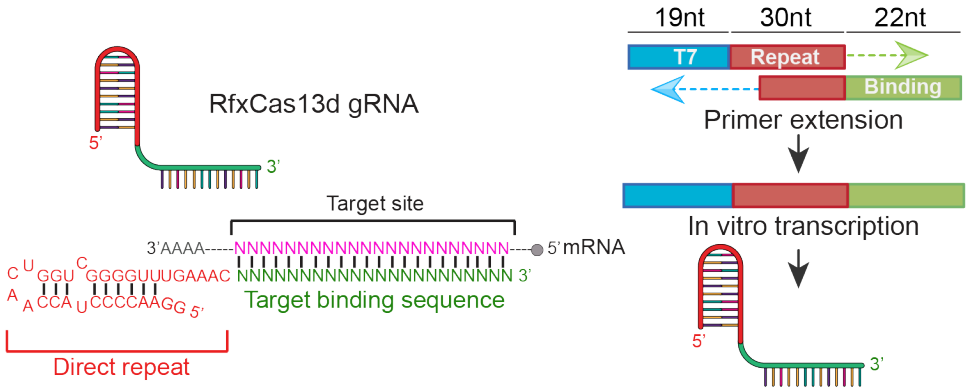
CRISPR-Cas13d induces efficient mRNA knock-down in animal embryos



Preparation of gRNA

Select the desired gene to target, paste the complete mRNA (including 5' UTR and 3' UTR) or the region that you might want to target (e.g. alternative exon) sequences into the RNA fold online tool (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi). Select minimum free energy (MFE) and partition function and then choose protospacers of 22 nucleotides (target sequences) with high accessibility (low base-pairing probability from minimum free energy predictions) (Lorenz et al., 2011). CRISPR-RfxCas13d system does not require PFS (PAM like) sequence in eukaryotic cells.



mRNA sequence:
Example of the coding sequence region that have been selected, the sequence is shown every 3 nucleotides to illustrate the codons and therefore, that the sequence selected is the positive sense strand.

xxx-xxx-xxx-NNN-NNN-NNN-NNN-NNN-NNN-Nxx-xxx-xxx-xxx-xxx

eg. -xxx-xxx-TTT-GGG-TGT-AGC-GTA-TGC-ACG-Gxx-xxx-xxx-xxx-xxx-
Selected sequence (spacer) is pasted as it is below (pink) upstream of the linker region (stem-loop of the gRNA, red). This sequence will serve as a reverse primer for gRNA amplification using a fill-in PCR approach.

NNNNNNNNNNNNNNNNNNNNGTTTCAAACCCCGACCAGTT

eg. TTT-GGG-TGT-AGC-GTA-TGC-ACG-GGTTTCAAACCCCGACCAGTT

RxfCas13d_universal: (Blue = T7 promoter and Red = RxfCas13d linker sequence)

TAATACGACTCACTATAGGAACCCCTACCAACTGGTCGGGGTTTGAAAC

Example:

Forward primer:
TAATACGACTCACTATAGGAACCCCTACCAACTGGTCGGGGTTTGAAAC

Reverse Primer:
NNNNNNNNNNNNNNNNNNNNGTTTCAAACCCCGACCAGTT

eg. TTT-GGG-TGT-AGC-GTA-TGC-ACG-GGTTTCAAACCCCGACCAGTT

PCR:

15 µL Nuclease-Free Water
5 µL Universal oligo (100 µM)
5 µL specific oligo (100 µM)
25 µL 2x Go taq master mix (Promega)

50 µL of total volume

(At least, 3 specific gRNA oligos can be amplified together as a pool)

PCR (30 cycles)

	Temperature (degree Celsius)	Time
Initial denaturation	94	4 min
Denaturation	94	30 sec
Annealing	60	40 sec
Extension	72	30 sec
Final extension	72	10 min
Store	4	

PCR product is purified using QIAquick PCR purification kit as given in the protocol book instructions.

1. Use binding buffer 5 volume to the sample in column (provided with kit). centrifuge at max speed of 13000g for 30 sec.
2. Wash 2 times with wash buffer (700 µL), centrifuge at max speed of 13000g for 30 sec.
3. Elute the sample, with nuclease free water in 30-50 µL volume by centrifuging the sample at max speed of 13000g for 30 sec.
4. Desired concentration by nanodrop should be 100-150ng/ µL.
5. It is recommended to run 1 µL in agarose gel (2%) to verify that the PCR has worked. The expected size should be approx. 70 bp.

Expected PCR sequence for the example:

TAATACGACTCACTATAGGAACCCCTACCAACTGGTCGGGGTTTGAAACCCGTGCA
TACGCTACACCCAAA

Use purified PCR product as a template for *in vitro* transcription reaction using AmpliScribe-T7-Flash transcription kit, Epicenter.

IVT Reaction:
x µL Nuclease-Free Water,
0.5-1 µg (Max 6.3 µL) PCR product
2 µL 10X Transcription Reaction Buffer
1.8 µL 100 mM ATP
1.8 µL 100 mM CTP
1.8 µL 100 mM GTP
1.8 µL 100 mM UTP
2 µL 100 mM DTT
0.5 µL RiboGuard RNase Inhibitor
2 µL T7 RNA Polymerase

20 µL Total reaction volume

1. Set the reaction for overnight, 12-16 h at 37ºC,
2. Digest the sample with 1 µL of Turbo DNase for 15 min at 37ºC
3. Add 80 µL of nuclease free water and precipitate gRNA using 1/10th volume of 3M sodium acetate and 3 volumes of ethanol 96-100% (molecular biology grade) for one hour at -80ºC.

4. Pellet down RNA by centrifuging at 13000g for 15 min 4ºC. A white pellet at the bottom of tube will be visualized.
5. Wash the pellet 2 times with 75% ethanol (13000g for 10 min 4ºC). Air dry the pellet for not more than 10 minutes. A white RNA pellet at the bottom of the tube will be visualized.
6. Reconstitute the RNA in desired volume of nuclease free water (30-50 µL).
7. Measure the concentration using qubit kit. it should be 400-600 ng/µL approximately. Final concentration needed for microinjections should be (300-600 ng/µL) in the injection mix. Aliquots with 10 µL of gRNAs were stored at -80ºC until use (they can be thaw and frozen 2-3 times without appreciable decreased in the activity).

It is recommended to run 1µL of the IVT into a clean RNase free agarose gel (2%) to visualize a single band. 15-20 mins are enough at 100 volts (Don't run for too long!), just need to visualize a single no-degraded band below 100 bp DNA marker.

Preparation of Cas13d mRNA

1. Take 2-5 µg of pT3TS-RfxCas13d (addgene #141320) plasmid and digest with *Xba*I enzyme as given below.

   2-5 µg of miniprep plasmid
   2 µL of 10X NEB CutSmart® Buffer
   1µL of *XbaI* enzyme (NEB)
   Add nuclease free water for 20µL reaction.

2. Digest the plasmid for 2 h. at 37ºC.
3. Run it on the agarose gel (0.8%), cut the linearized expected size band using new sharp scalpel blade.
4. Using Qiagen Gel extraction kit to purify the linearized plasmid.
5. Use 5 volume of the binding buffer to the extracted gel piece to the tube.
6. Heat at 50-55ºC for 10 mins with shaking until it gets fully dissolved.
7. Add the dissolved product to the columns provided with the kit and centrifuge at the max. speed of 13000g for 30sec.
8. Discard the flow through and add if there is some amount of dissolved product left and centrifuge, at the speed of 13000g for 30sec.
9. Wash 2 times with wash buffer (700µL), centrifuge at max speed of 13000g for 30 sec.
10. Elute the sample, with nuclease free water in 30-50µL volume by centrifuging the sample at max speed of 13000g for 30 sec.
11. Desired concentration by nanodrop should be 150-200ng/µL (Qubit reading).
12. It is recommended to run it in an agarose 0.8% gel to verify the linearization.
13. Use T3 Message Machine kit (Ambion, AM1348) for mRNA synthesis, incubate for 2 hrs (we perform 2-3 reactions separately that we purify together to increase final concentration).

IVT reaction:

10 µL 2X NTP/CAP
2 µL 10X Reaction Buffer
6 µL of linearized template
2 µL Enzyme Mix
Incubate for 2 hours at 37 degree Celsius.

14. Digest it with 1 µL of TurboDNase (Ambion) for 15 min.
15. Purify the RNA using an RNeasy Mini kit (QIAGEN) following manufacter recommendations. 40-60 µL from 2-3 reactions are pooled to increase final yield and the treated as one single sample. We recommend the final elution in 30-40 µL nuclease free water.
16. Qubit yield should be 800-1200ng/µL approximately.
17. Run in a 1% agarose gel to check for RNA integrity.

Injection mix:

After taking the qubit readings for both gRNAs and Cas13d mRNA, make a final reaction volume in 10µL. In injection mix cas13dmRNA concentration for zebrafish (200-300ng/ul) and gRNA concentration (300-600ng/µL). Inject 1-2 nL of injection mix into the one cell stage zebrafish embryos.

RfxCas13d Protein expression

1. *Escherichia coli* Rosetta™ (DE3) competent cells are transformed with the pET28B-RfxCas13d-His plasmid and plated on LB broth supplemented with 25 µg/mL Kanamycin and 15 µg/mL Chloranphenicol.

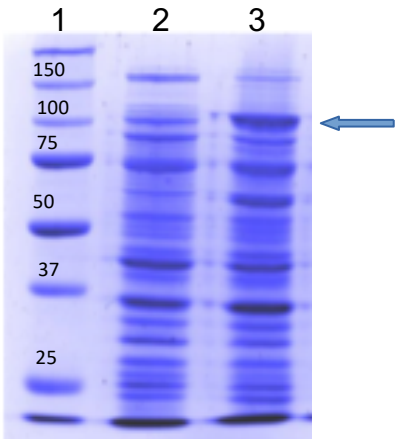
2. Pick a colony and inoculate it into 10 ml of LB medium containing 25 µg/mL Kanamycin and 15 µg/mL Chloranphenicol and incubate it with shaking at 37°C, overnight.

3. The next morning, dilute 1:100 cells into 500 ml fresh culture medium and grow the culture with shaking at 37°C until the OD600 reaches 0.5. At this point, transfer 1 ml of the culture to an 1.5 ml tube and centrifuge it to harvest cells. Store pellet at -20ºC until analysis.

4. Add IPTG to a final concentration of 0.1 mM to the rest of the culture and grow the culture with shaking at 37°C for 3 hours. It normally shows an OD600 1.5-2.0

5. After the induction, harvest cells 15 min at 8.000 g at 4ºC, wash with 20 mM TrisHCl pH 7.6 and store the pellet at -80ºC for protein purification. To analyze the expression, before harvesting the cells, transfer 1 ml of the culture and centrifuge it to precipitate the cells.

6. To analyze protein expression, suspend the 1 ml cell pellet in 25 µL H_2O/DO600. Mix sample with the same volume of 2xSDS Sample Buffer (Tris-HCl 160 mM pH 8, glycerol 20% v/v, Bromophenol blue 0.1%, SDS 2%, DTT 200mM) heat at 85ºC for 5 min and subject 5 µL of each sample to a 10% SDS-PAGE. A more abundant band must appear (Lane 3) in the rage of 100 KDa (Lane1) when comparing with cells before induction (Lane 2). See figure below.



Protein Purification

Protein purification was performed with a HisTrap FF column (GE Healthcare) in a ÄKTA pure system (GE Healthcare).

1. Suspend cell pellet in 25 mL Lysis buffer (50 mM HEPES·KOH pH 7.5, 500 mM KCl, 10% v/v glycerol, 1mM DTT and 10 mM imidazole) on ice

2. Sonicate cells during 10 min at 40% amplitude with cycles of 5´´on-10 ´´off, placing the sample on ice

3. Centrifuge cell lysate at 10.000 g during 15 min and discard pellet. Pass the supernatant through a 0.22 µm cellulose acetate filter.

4. Affinity Chromatography was run at 1ml/min at 10ºC. Equilibrate column with 5 column volume (CV) of Lysis buffer before sample application, apply sample at 0.5 mL/min and wash column with 20 CV of Lysis buffer. Elution was performed with a two-step elution protocol; first with 5 CV of 10% Elution buffer (50 mM HEPES·KOH pH 7.5, 500 mM KCl, 10% v/v glycerol, 1mM DTT and 500 mM imidazole) and second with of 20 CV gradient until 50% of Elution buffer and fractions of 1.5 ml were collected.

5. To check which fractions contains the purest RfxCas13d, 10 µL of each fraction were checked with 10% SDS-PAGE. Typically, fractions from 8 to 14 were selected (Fig, S4A), pooled and dialysed against 1 L of Dialysis buffer (50 mM HEPES·KOH pH 7.5, 250 mM KCl, 1 mM DTT and 10% glycerol) over night at 4ºC.

6. After dialysis, sample was concentrated by ultrafiltration with a 30K Amicon Ultra-15 (Millipore). Protein concentration was checked during ultrafiltration with the RC-DC protein assay kit (Bio-Rad) and when it reached 3 µg/µL (approximately 10 fold volume concentration), ultrafiltration was stopped. Single-use aliquots with 5 µL of Cas13d were stored at -80ºC until use.

7. Purity and final concentration was checked by loading a 10% SDS-PAGE with known amounts of Bovine Serum Albumin (typically 0.14, 0.7 and 1.4 µg) and several dilutions of RfxCas13d. A colorimetric stain was applied and a densitometric scanning was performed to quantify protein bands.

Injection mix protein:

RfxCas13d protein (3 µg/µL) and gRNAs (300-1000 ng/µL) were injected in two rounds (1 nL per embryo in each round) to maximize the amount of protein and gRNA per injection. Alternatively, RNPs can be assembled in dialysis buffer by mixing 5 µL of RfxCas13d (3 µg/µL) and 0.5 µL gRNAs (3.2 µg/µL) and co-injected in one round (final concentration in the embryo: ~ 2.75 ng of RfxCas13d and ~ 300 pg of gRNAs). This can be done with chemically synthesized gRNAs that are highly concentrated. Inject 1-2 nl of mix into the one cell stage zebrafish embryo.