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CRISPR/Cas7-11 tool for RNA targeting in *Xenopus laevis*

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We use this protocol and it's working

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Abstract

CRISPR/Cas systems are defense mechanisms in prokaryotic cells to induce the cleavage and degradation of nucleic acids derived from bacteriophages. Among the different classes, only type III (Cas7) and type VI (Cas13) systems target RNA. Recently, engineered Cas7-11 (a single protein resulting from the fusion of putative Cas11 domains with multiple Cas7 subunits derived from subtype III-D) was used in both mammalian cells and zebrafish. This technology relies on the use of guide RNAs to target a specific mRNA for cleavage and degradation. However, the applicability of this RNA-targeting system in other animals remains largely underexplored. In this protocol, we provide a comprehensive workflow outlining the implementation of this tool in *Xenopus* embryos, especially focused on guide design, synthesis, Cas7-11 mRNA, microinjection and knockdown validation. We provide some important tips and troubleshooting for efficient, specific and reliable downregulation of genes in this model organism.



Protocol materials

✂ mMESSAGE mMACHINE[®]; T7 Transcription Kit **Thermo Fisher Catalog #AM1344**

✂ Qubit[®]; RNA BR Assay Kit **Thermo Fisher Catalog #Q10210**

✂ Q5 Hot Start High-Fidelity DNA Polymerase - 500 units **New England Biolabs Catalog #M0493L**

✂ MinElute Gel extraction kit **Qiagen Catalog #28604**

✂ Monarch RNA Cleanup Kit (50 ug) **Catalog #T2040**

✂ NotI-HF - 500 units **New England Biolabs Catalog #R3189S**

✂ mMESSAGE mMACHINE[®]; SP6 Transcription Kit **Thermo Fisher Catalog #AM1340**

✂ RNeasy mini kit **Qiagen Catalog #74106**

Safety warnings

- ! - CRITICAL: gRNAs should be purified via small RNA purification kits.
- CRITICAL: Use a spectrophotometer (Nanodrop) for gRNA quantification will overestimate the gRNA concentration.
- CRITICAL: Run a bleach gel (1% commercial bleach/ 2% agarose) to check for the integrity of the gRNAs.

Designing gRNAs for the 3' UTR of the mRNA target sequence (*in silico*)

2h

- 1 Search for your gene in Xenbase (<https://www.xenbase.org>) (Fisher et al, 2023). In "Nucleotides, select all the transcripts and paralogues for your gene and download the complete mRNA sequences (including UTR's);
- 2 Perform an alignment between different transcript variants and/or paralogues and identify the 3'UTR;
- 3 To design the most promising guides, use a platform (<https://cas13design.nygenome.org/>) that provides optimized spacers to target transcripts in the human transcriptome, model organisms and viral RNA genomes using CRISPR-Cas13 (Wessels et al, 2020; Guo et al, 2021);

Note

This platform relies on a machine learning-based "on-target" model that takes into consideration several parameters, such as the crRNA-fold energy, the local target C context and the upstream target U context (Wessels et al, 2020);

- 3.1 Go to "Design costum gRNAs", since *Xenopus laevis* is not yet included in the list of model organisms;
- 3.2 We started by designing guides for the transcript with the longest 3'UTR. For sequences < 500 nt, copy and paste the sequence where you want to design the guides. If a sequence is larger than 500 nt, use "upload multiple single-entry fasta files", and the results will arrive at the email within a few minutes;
- 3.3 From the list provided, select top 3 spacers positioned in quartile 4 (Q4) with a minimum distance of 50 nt between them to avoid potential competition between gRNAs for target binding (Hernandez-Huertas). Each spacer will have 23 nt;
- 3.4 We recommend evaluating the most promising spacer sequences by aligning them with various transcript variants and/or paralogues and choosing those that show conservation across these sequences;
- 3.5 *Optional but recommended:* Analyse if the selected spacers have potential off-target effects. For this purpose, perform a BLAST search of the spacers against the RNA-seq data of the *Xenopus laevis* database in NCBI. Consider a direct off-target if it has up to four mismatches with the target (Kushawah et al, 2020). To further evaluate the off-target risk, analyse whether the potential off-target is expressed in the tissue of interest/developmental stage.

Note

According to a study using CRISPR Cas13 (Kushawah *et al*, 2020), guides for the CDS are more efficient at inducing a phenotype than guides for the UTR's. However, a great disadvantage in designing guides for the CDS is the impossibility of performing rescue experiments by reintroducing the mRNA of the gene being targeted. If that is the case, you can perform the rescue with a version that is not targeted by your guides (for instance, creating a CDS resistant to your guides using silent mutations or expressing the CDS of another species that cannot be targeted by your guides).

Preparation of the template PCR for gRNA transcription

2h

- 4 Follow the steps of a fill-in PCR (Hernandez Huertas) to generate the DNA template for gRNA production. The following primers should be used:
 - a) Universal primer: containing the direct repeat (DR) for the CAS7-11 interaction used in Özcan *et al.*, 2021 (Özcan *et al*, 2021) and the T7 minimal promoter;
 - b) Specific primer: This primer contains the complementary region that binds the linker and a sequence that should correspond to the mRNA target region (the same as the reverse complement of the guides);

Note

We strongly recommend to simulate the PCR with your designed primers using to SnapGene® software (Go to "Actions" and then "PCR") or other tool that allows to simulate your PCR to avoid any error in the design of the specific primer.

- 5 Use a mixture of universal primer and specific primers. We performed PCR using a pool of three different gRNA-specific primers at equimolar concentrations (3.3 μ M of each primer per  50 μ L of PCR, which corresponds to approximately 10 μ M in total).

Note

Alternatively, gRNAs can be individually produced by performing separate PCRs for each gRNA template using 10 μ M of the specific primer and 10 μ M of the universal primer. gRNAs produced should be mixed at equal concentration in the injection solution.

Note

We recommend performing at least three PCRs and mixing them to increase the amount of DNA produced after purification. Negative controls with universal or specific primers alone can be run to ensure that the PCR bands are correct.

6 We performed PCR using

3m 40s



Q5 Hot Start High-Fidelity DNA Polymerase - 500 units **New England Biolabs Catalog #M0493L**

, but other DNA polymerases can be used (just following the proper manufacturer's instructions). In our case, the PCR conditions were as follows: (i)  98 °C for  00:00:30 ; (ii)  98 °C C for  00:00:10 ; (iii)  61 °C for  00:00:30 ; (iv)  72 °C for  00:00:30 (29× cycles, steps ii–iv); and (vi)  72 °C for  00:02:00 .

7 Mix all three PCR products and run  5 µL on a 2% agarose gel. A band less than 100 bp (75 bp) should be observed;

8 Purify PCR products using a  MinElute Gel extraction kit **Qiagen Catalog #28604** or other PCR purification kit following the manufacturer's instructions.

Note

The kit we used allows a good yield to be obtained in a small volume of elution. The minimum amount of nuclease-free water recommended for elution was used to increase the concentration of the sample for the posterior step of *in vitro* transcription.

***In vitro* transcription of the gRNAs**

5h

9 We optimized the protocol for gRNA synthesis and purification as follows:

9.1 Use 500 ng of the purified PCR template per *in vitro* transcription;

9.2 Use

4h

 mMESSAGE mMACHINE[®]; T7 Transcription Kit **Thermo**
Fisher Catalog #AM1344

following the manufacturer's instructions. We performed transcription at  37 °C for

 04:00:00 ;

9.3 Add  1 µL of TURBODNase (2 U/mL) (provided with the transcription kit) and incubate at  37 °C for  01:00:00

1h



Note

CRITICAL: gRNAs should be purified via small RNA purification kits. We used a  Monarch RNA Cleanup Kit (50 ug) **Catalog #T2040** to purify the RNA following the instructions, but with the adjustment for smaller RNAs and RNA with secondary structures. Thus, we diluted our samples with two volumes of ethanol instead of one in Step 2 of the manufacturer's protocol. Elution should also be performed in the minimum volume of nuclease-free water recommended.

Note

CRITICAL: The use of a spectrophotometer (Nanodrop) for gRNA quantification will overestimate the gRNA concentration (Hernandez-Huertas). A Qubit RNA BR (Broad-Range) assay kit (Invitrogen) is crucial for accurate quantification of these small RNA fragments.

9.4 Always run a bleach gel (1% commercial bleach/2% agarose) to check for the integrity of the gRNAs. The use of parallel samples whose concentrations were previously known could also be a good quality control.

Note

Do always three to four transcriptions and mix them to have enough amount for all the experiments, using the same batch of gRNA. Expected yields can vary because of their composition and secondary structures (Hernandez-Huertas), but in general, concentrations between 100 ng/µl and 500 ng/µl can be obtained using this method.

**Note**

We always recommend storing gRNAs at -80 °C in several aliquots (e.g. 5 µL) to avoid freeze–thaw cycles.

***In vitro* transcription of Cas7-11 msfGFP mRNA**

9h

- 10 Use 6 µg of the pCS2+ huDisCas7-11-msfGFP plasmid produced in this study to digest the mixture for 04:00:00 with

4h

NotI-HF - 500 units **New England Biolabs Catalog #R3189S** .

Note

We usually use a 100 µL digestion volume to increase the chance of obtaining good HuDisCas7-11-msfGFP mRNA concentrations after purification.

- 11 Purify the products with a MinElute Gel extraction kit **Qiagen Catalog #28604**) or other kit for purification of DNA fragments.

4h

- 12 Transcribe using 2 µg and the

mMESSAGE mMACHINE[®]; SP6 Transcription Kit **Thermo Fisher Catalog #AM1340**

; Incubate at 37 °C for 04:00:00

- 13 Proceed with digestion with TURBO DNase 01:00:00 at 37 °C);

1h

- 14 Proceed with the clean-up; we used an RNAeasy mini kit **Qiagen Catalog #74106** ;

- 15 Quantify mRNA via a NanoDrop™ or

Qubit[®]; RNA BR Assay Kit **Thermo Fisher Catalog #Q10210** and run on a gel bleed gel (as described previously);



Microinjection of embryos

2h

- 16 In this study, we injected the dorsal and ventral blastomeres (right or left) of 8 cell-stage embryos to target the neural crest and the two ventral vegetal blastomeres of 4 cell-stage embryos to target the ventral mesoderm.
- 17 Guides were injected at 160 pg/blastomere and mRNA from Cas7-11 msfGFP was injected at a concentration of 800 pg/blastomere under all the conditions;

Note

We recommend performing an initial titration to determine the concentration of gRNA and Cas7-11 msfGFP mRNA that should be used without affecting the viability of the embryos or the biological process.

Validation of mRNA knockdown

2w

- 18 To validate the efficiency of the knockdown, we propose the following complementary or alternative experiments:
 - 18.1 SqRT-PCR or qPCR (2 weeks): Analysing the mRNA levels of a target gene through semi quantitative RT-PCR analysis is a fast way to address the knockdown efficiency if the depletion is successful and evident. For a more quantitative analysis, use qPCR with specific primers for the target gene;

In situ hybridization (2 weeks): This is a complementary way to check for the efficiency of depletion and requires designing specific probes for the gene you want to analyse or using probes already published and validated

RNA-seq (variable time): This type of analysis will reveal not only the efficiency of depletion but also how the whole transcriptome is affected.
- 19 Always perform rescue experiments for the gene you are targeting for phenotype validation. If guides are designed to target UTR's, a rescue experiment can be performed by simply reintroducing the coding sequence of a gene; titration of the amount of mRNA should be performed to avoid dominant effects caused by overexpression.

Troubleshooting

- 20 **Problem: Low gRNA and Cas7-11 mRNA concentrations**

- a) If a low gRNA concentration is obtained, check the design of the primers, ensuring that the T7 minimal promoter is included in the “universal primer”;
- b) For Cas7-11 mRNA, check the linearization efficiency of the plasmid;
- c) If low gRNA and/or Cas7-11 mRNA levels are obtained, the amount and purity of the template used for *in vitro* transcription should be checked, as these aspects are crucial for successful *in vitro* transcription;
- d) For gRNA purification, use a kit that allows the purification of low-quality fragments;
- e) Alternatively, commercially-synthesised gRNAs (Moreno-Sánchez et al, 2025) can be used to reduce the variability related with guide concentration and quantification, as well as associated toxicity effects. This approach can also save time and lower overall costs;

21 **Problem: Deficient RNA depletion and weak/absent phenotype**

- a) Check the design of your guides;
- b) Do not use poly(A) tailing in your gRNAs, as this can critically alter the structure of your guides;
- c) For linearization of the plasmid to produce Cas7-11 mRNAs, a single cut restriction that cuts after poly(A) is used; in this case, poly(A) is essential to confer stability and enhance translation efficiency *in vivo*;
- d) The concentration of each gRNA must be checked via a reliable method (with a Qubit fluorometer). As discussed before, this is a critical aspect to consider because the guide concentration can be overestimated by a NanoDrop spectrophotometer;
- e) Run the samples (e.g. 500 ng) in 1% commercial bleach/2% agarose as suggested before to check the integrity of the gRNAs and Cas7-11 mRNA; if possible, a sample of gRNAs or Cas7-11 mRNA that worked previously should be run in parallel;
- f) Always ensure that you are using RNase-free material during the transcription and purification processes and avoid freeze–thaw cycles;
- g) If an embryo has a low survival rate, a lower concentration of gRNAs and/or Cas7-11 mRNAs should be tested;

- h) The efficiency of targeted injections should always be carefully checked. Use a fluorescent dye (e.g. dextran red) to confirm the efficiency of the injection (as the msfGFP of the Cas7-11 signal can sometimes be faint owing to embryo autofluorescence and/or the ubiquitous distribution of Cas7-11 msfGFP in cells);
- i) If the signal of an injection marker is faint, it might be possible that the injection conditions (e.g., technical aspects related to the needle) are not optimal. Additionally, a high amount of mRNA may be toxic to cells, resulting in death, fewer injected cells and consequently a less penetrant phenotype.

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