Protocol

Confirming Antibody Specificity in *Xenopus*

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Verifying that a new antibody recognizes its target can be difficult. In this protocol, expression of a target protein in *Xenopus* embryos is either knocked down using CRISPR–Cas9 technology (for zygotic proteins) or enhanced by microinjection of a synthetic mRNA (for maternal proteins). Western blotting analysis is then performed. If the antibody recognizes the target protein, the western blot will show a relatively weak band for CRISPR-injected embryos and a relatively strong band for RNA-injected embryos. This represents a straightforward, powerful strategy for confirming antibody specificity in *Xenopus*.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous materials used in this protocol.

RECIPES: Please see the end of this protocol for recipes indicated by <R>. Additional recipes can be found online at <http://cshprotocols.cshlp.org/site/recipes>.

Reagents

Agarose gel electrophoresis reagents (i.e., 1%–2% agarose gels, loading dye, running buffer, and ethidium bromide)

Antibody to test

See Protocol: **Raising Antibodies for Use in *Xenopus*** (Piccinni and Guille 2020).

Cysteine (2% [w/v], pH 7.8; Sigma-Aldrich C7352)

Embryo extraction buffer <R>

Ficoll (3% [w/v], prepared in 1× MBS; Sigma-Aldrich GE17-0300-50)

Freon (1,1,2-trichloro-1,2,2-trifluoroethane; Fisher Scientific T1781)

Modified Barth's saline (MBS) (1×, pH 7.8) <R>

Nuclease-free dH₂O (Fisher Scientific 15303711)

Reagents for CRISPR analysis only (see Steps 1–9 and Steps 25–33)

Cas9 protein (EnGen Cas9 NLS, *Streptococcus pyogenes* [NEB M0646])

DNeasy Blood & Tissue Kit (QIAGEN) or other appropriate kit to prepare genomic DNA from embryos

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gDNA oligo (100 μ M, dissolved in H₂O)

MEGashortscript T7 Transcription Kit (Thermo Fisher Scientific AM1354)

MgCl₂ (500 μ M)

NEBuffer2 (NEB B7002S)

PCR master mix (2 $\times$; see Table 1)

PCR primers designed to amplify 600–800 bp containing the CRISPR target site close to the amplicon's center (for Step 26)

Q5 Hot Start High-Fidelity 2 $\times$ Master Mix (NEB M0491S)

T7 Endonuclease I (NEB M0302)

Universal 3' gRNA template (5' AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAAC
GGACTAGCCTTATTTAACTTGCTATTCTAGCTCTAAAAC 3') (100 μ M)

Reagents for overexpression analysis only (see Steps 10–17)

Plasmid encoding the target ORF surrounded by the globin UTRs driven by a T7, SP6, or T3 promoter (e.g., pCS2 [Rupp et al. 1994])

Such plasmids are available from the European Xenopus Resource Centre (EXRC), which also holds ORFeomes (representing more than 14000 genes).

Restriction enzyme appropriate to cut plasmid encoding the target ORF (see Step 11)

SuperScript kit (Ambion) for long transcripts

Choose a kit based on which promoter is in the plasmid encoding the protein.

SDS-PAGE reagents (i.e., sample buffer, gel, and running buffer; see Step 35)

Western blotting reagents (see Step 36)

Xenopus eggs, fertilized (see Guille 1999)

*If targeting a specific protein with CRISPR, consider your choice of species (see Step 1). If *Xenopus tropicalis* is available, its rarity of gene duplications makes it preferable to *Xenopus laevis* for the ~60% of genes that have homeologs in the latter.*

Equipment

Access to a sequencer (see Step 33)

Agarose gel electrophoresis apparatus

Benchtop centrifuge

Filter tips for pipettes

Flame-polished glass Pasteur pipettes, with a bore slightly larger than embryos

Glass needles for use in a microinjector

Incubators (18°C for *X. laevis* embryos; 25°C for *X. tropicalis* embryos)

Injection dish (mesh or agarose; prepared in house as described by Guille [1999])

Microinjector

Parafilm

PCR clean-up columns (for overexpression analysis only; see Steps 10–17)

Programs for designing sgRNAs (e.g., CRISPRscan; see Step 1) and TIDE analysis (see Step 33)
(for CRISPR analysis only)

SDS-PAGE gel electrophoresis apparatus

SigmaSpin Sequencing Reaction Clean-Up columns (for CRISPR analysis only; see Steps 1–9)

Spectrophotometer capable of measuring microliter volumes

Sterile microcentrifuge tubes

Sterile PCR tubes

Thermocycler

Vortex mixer

Western blot transfer and imaging systems

METHOD

Preparation of Single Guide RNA (sgRNA) or Synthetic RNA

To synthesize sgRNA for knocking down a target protein in *Xenopus* embryos by CRISPR, begin with Steps 1–9. To prepare synthetic RNA for overexpressing a target protein in *Xenopus* embryos, begin with Steps 10–17. The former strategy is best for zygotic proteins, whereas the latter is best for maternal proteins (see Discussion).

Design and In Vitro Transcription of sgRNA (for CRISPR Only)

This is best suited to zygotic proteins.

1. Design sgRNAs.

This can be done using a variety of programs. We have had great success using CRISPRscan (<http://www.crisprscan.org>) to target the first or second translated exon of the gene encoding the protein against which the antibody was raised. If working in *X. laevis*, then both homeologs will almost certainly need to be targeted; it is, however, worth checking that both of the genes are transcribed at significant levels by using the expression tab on Xenbase. We normally design three sgRNAs for each gene because there is variability in their effectiveness.

2. Prepare the PCR mix by combining reagents as outlined in Table 1. Remove 3 μ L before reaction and place on ice.

3. Anneal and extend the sgRNA template (ssDNA) oligos in a thermocycler using the program outlined in Table 1.

4. Run pre- and postreaction (3- μ L) samples on a 2% agarose gel using standard procedures.

Ensure that the postreaction band is much stronger with slightly lower mobility compared to the prereaction band.

5. Perform the in vitro transcription reaction according to the MEGashortscript T7 Transcription Kit instructions, using 2 μ L of the PCR from Step 3. Ensure that the reaction is set up at room temperature and not on ice.

Because only 2 μ L of the PCR is used, the remaining DNA can be stored at -20°C .

6. Remove small molecules from the reaction by spinning through a SigmaSpin Sequencing Reaction Clean-Up column following the manufacturer's instructions.

7. Run 3 μ L of sgRNA plus loading buffer on a 2% agarose gel for 15 min.

A single band should be visible.

8. Quantify the sgRNA by measuring the A_{260} of 1 μ L of sgRNA using a suitable spectrophotometer.

9. Store the sgRNA at -80°C in 300-ng aliquots.

Proceed to Step 18.

TABLE 1. Components and steps to synthesize dsDNA from ssDNA by an annealing-extension reaction

PCR components	PCR master mix (2 $\times$)	50 μ L		
	gDNA oligo (100 μ M)	2 μ L		
	Universal gRNA template (100 μ M)	2 μ L		
	MgCl ₂ (500 μ M)	2 μ L		
	Nuclease-free dH ₂ O	44 μ L		
	Reaction total	100 μ L		
Reaction steps	1 cycle	Initial denaturing	94 $^{\circ}\text{C}$	5 min
	20 cycles	Denaturing	94 $^{\circ}\text{C}$	20 sec
		Annealing	58 $^{\circ}\text{C}$	15 sec
		Extension	68 $^{\circ}\text{C}$	15 sec
	1 cycle	Final extension	68 $^{\circ}\text{C}$	5 min
	1 cycle	Hold	4 $^{\circ}\text{C}$	

Preparation of Synthetic RNA (for Overexpression Analysis Only)

This is best suited to maternal proteins.

10. Prepare plasmid encoding the target ORF surrounded by the globin UTRs driven by a T7, SP6, or T3 promoter (e.g., pCS2 [Rupp et al. 1994]).
11. Linearize 10 µg of plasmid with a unique restriction enzyme that cuts downstream from the synthetic poly(A) site or polyadenylation signal (depending on the plasmid).
12. Purify the DNA using a PCR clean-up column and resuspend it at 1 mg/mL in nuclease-free water.
13. Use this DNA as a template to synthesize RNA with the appropriate Ambion SuperScript kit following the manufacturer's instructions. Ensure that the reaction is set up at room temperature.
14. Remove small molecules from the reaction by spinning through a SigmaSpin sequencing reaction cleanup column following the manufacturer's instructions.
15. Run 1 µL of RNA on a 1% agarose gel.
A bright band should be visible. If multiple bands are seen, these most often represent conformers of the RNA and can be resolved to a single band in a denaturing gel.
16. Quantify the synthetic RNA by measuring A_{260} of 1 µL of the sample using a suitable spectrophotometer.
17. Store the RNA in single-use 2-µL aliquots at -80°C .

Proceed to Step 18.

Injection into Fertilized *Xenopus* Eggs

18. Calibrate an injection needle to inject 4 nL of liquid. Keep the needle tip hydrated in a dish of nuclease-free dH_2O when it is not in use.
19. When fertilized *Xenopus* eggs have turned their animal poles uppermost, add a solution of 2% (w/v) cysteine (pH 7.8) prepared in water. Agitate the embryos gently for 5 min at room temperature until they have been dejellied, and then wash them five times at room temperature in $1\times$ MBS.

Each wash should be of sufficient length to allow the embryos to settle after swirling to mix them with the MBS.

20. Prepare the injection solution.

For CRISPR–Cas9

- i. Prepare the injection mixture by combining 16 µM Cas9 protein, 200 ng sgRNA, and nuclease-free H_2O to 4 µL.
A second injection mix without sgRNA may be prepared and injected as a control.
- ii. Mix gently, and centrifuge at full speed in a benchtop centrifuge for 1 min at room temperature.

For synthetic RNA

- iii. Prepare 1 µg of synthetic RNA in 4 µL of nuclease-free H_2O .
- iv. Mix gently, and centrifuge at full speed in a benchtop centrifuge for 1 min at room temperature.
21. Place a 1- to 3-µL droplet of injection solution onto a square of Parafilm, and fill the needle from the droplet.
22. Fill an injection dish with 3% (w/v) Ficoll in $1\times$ MBS. Transfer 50–100 embryos to the injection dish using a precut glass pipette.

23. Inject the solution into the animal pole of each embryo.
24. Once injected, transfer the embryos into an incubator at the appropriate temperature and wait 3–4 h before transferring them to 0.1× MBS and removing damaged embryos.

Continue with Step 25 if the CRISPR analysis was performed, or proceed to western blotting (Steps 34–36) if synthetic RNA was injected for overexpression of the protein of interest.

T7 Endonuclease Assay and TIDE Analysis (for CRISPR Only)

The T7 endonuclease assay is used to confirm specific cutting by Cas9, validating the sgRNA used. TIDE analysis gives an approximate efficiency reading for the cutting of a specific site using a specific sgRNA.

T7 Endonuclease Assay

25. Prepare genomic DNA from embryos at stage 12 or later. Use an appropriate kit and follow the manufacturer's instructions. Store the genomic DNA at -20°C .
We use QIAGEN's DNeasy Blood & Tissue Kit.
26. Perform PCR by combining 100 ng of genomic DNA, 10 μM of forward primer, 10 μM of reverse primer, 25 μL of Q5 Hot Start High-Fidelity 2× Master Mix (which includes enzyme), and nuclease-free dH_2O to 50 μL . Include a reaction with DNA from an uninjected embryo as a control. Amplify using a basic amplification program with primer-specific annealing temperatures.
27. Run 3 μL of the amplicon mixed with 1 μL of loading solution on a 1% agarose gel.
28. Use spectrophotometry to measure the amplicon concentration.
29. Combine 2 μL of NEBuffer 2, ~200 ng of amplicon, and nuclease-free dH_2O for a final volume of 19 μL .
30. Denature the amplicon for 5 min at 95°C . Reanneal by cooling to 25°C at a rate of $0.1^{\circ}\text{C sec}^{-1}$ in a thermocycler, and then hold at 4°C .
31. Add 1 μL of T7 endonuclease I and incubate for 15 min at 37°C .
32. Visualize the products of the digestion on a 1.5% agarose gel.

T7 endonuclease will only cut at indels. Successful cutting will show two bands on a gel (or, if the cut site is in the center of the amplicon, there will appear to be one band at one-half the size of the amplicon).

TIDE Analysis

33. Sequence the amplicon obtained from Step 26 and analyze using TIDE, which uses algorithms to distinguish the different sequences that occur after the CRISPR cut site to predict where and how often the main indels have occurred (<https://tide.deskgen.com/>).

Once cut site digestion has been achieved at a good efficiency rate, proceed to western blot experiments (Steps 34–36) to test antibodies.

Western Blot Analysis

To choose which developmental stages to target for the western analysis, use the mRNA expression information on Xenbase as a guide. Although it is clear that translational control is used in early development, using stages at which mRNA levels are highest is the best starting point. This method is described in detail in Guille and Robinson (1999).

34. Prepare a crude protein extract.
 - i. For each experimental condition, gather 25 embryos.
 - ii. Add 250 μL of embryo extraction buffer, and pipette up and down with a 1-mL pipette until the homogenate is uniform.
 - iii. Add an equal amount of Freon, and vortex for 30 sec.

FIGURE 1. Western blot analysis of Fli1 protein overexpression in *Xenopus laevis* embryos. One-cell-stage *Xenopus laevis* embryos were injected with either H₂O (as a control; lane 1) or increasing amounts of *fli1* mRNA (lanes 2–5) and then allowed to develop until stage 17. After the embryos were homogenized, the proteins were Freon-extracted, separated by SDS-PAGE, and transferred to a nitrocellulose membrane. The western blot was probed with a 1/500 dilution of Fli1 anti-serum. After removal of the primary antibody, the blot was incubated with a 1/5000 dilution of donkey anti-sheep secondary antibody conjugated to horseradish peroxidase. The blot was developed using ECL Prime Western Blotting Detection Reagent (GE Healthcare). The band corresponding to Fli1 protein increases in intensity with increasing *fli1* mRNA injection amounts. The last lane (lane 6) is the protein from a *fli1* in vitro translation (Promega), showing that the Fli1 protein produced by in vitro translation is the same size as Fli1 protein in *Xenopus laevis* embryos.

- iv. Centrifuge the sample at 21,000g for 3 min at 4°C.
 - v. Collect the upper (protein) phase.
35. Separate proteins by SDS-PAGE, loading 0.5–1 embryo equivalent of crude protein extract per well in the SDS-PAGE sample buffer.
 36. Follow standard western blotting protocols to test the specificity of the antibody of interest.

Noninjected embryo samples should produce a band at the size expected for your protein of interest. If the antibody is detecting the target protein, CRISPR-injected embryos will show a weaker band, and the RNA-injected embryos will show a stronger band. For an example of the latter, see Figure 1.

DISCUSSION

Once an antibody has been raised against a target protein (see Protocol: **Raising Antibodies for Use in *Xenopus*** [Piccinni and Guille 2020]), various strategies can be used to verify that the new antibody recognizes its target. Sometimes using immunoprecipitation, tryptic digest, and mass spectroscopy of the target protein is the only convincing approach (Persson et al. 2017). In a well-established model like *Xenopus*, however, inducing loss or gain of function of the target protein in embryos followed by western blotting is a straightforward, powerful alternative strategy. Target protein can be knocked down using either CRISPR–Cas9 technology (Nakayama et al. 2014), as described here, or an anti-sense morpholino oligonucleotide (AMO; Heasman et al. 2000). Both have good penetrance when the target is not maternal. CRISPR–Cas9 is cheaper than AMO, and it is straightforward to confirm that insertion or deletion of sequence at the target site has occurred by using sequencing or T7 endonuclease. However, for AMOs targeting translation, there is no such simple positive control. For these reasons, knockdown by CRISPR–Cas9 is described here. For maternal proteins, microinjection of a synthetic mRNA encoding the target to increase its expression is preferable (Guille 1999; see Fig. 1).

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RECIPES

Embryo Extraction Buffer

10 mM HEPES (pH 8.5)
2 mM MgCl₂
1 mM EDTA
10 mM β-glycerophosphate
1 mM dithiothreitol (DTT)

Store for up to 1 wk at 4°C.

Modified Barth's Saline (MBS) (1×, pH 7.8)

Reagent	Quantity (for 1 L)	Final concentration (1×)
NaCl	5.143 g	88 mM
KCl	0.075 g	1 mM
MgSO ₄	0.120 g	1 mM
HEPES	1.192 g	5 mM
NaHCO ₃	0.210 g	2.5 mM
CaCl ₂ , dihydrate	0.103 g	0.7 mM
H ₂ O	to 1 L	

Adjust the pH to 7.8 with 10 M NaOH and sterilize by autoclaving. Store at room temperature indefinitely.

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