Protocol

RNAi-Mediated Loss of Function of *Xenopus* Immune Genes by Transgenesis

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Generation of transgenic frogs through the stable integration of foreign DNA into the genome is well established in *Xenopus*. This protocol describes the combination of transgenesis with stable RNA interference as an efficient reverse genetic approach to study gene function in *Xenopus*. Initially developed in the fish medaka and later adapted to *Xenopus*, this transgenic method uses the I-SceI meganuclease, a "rare-cutter" endonuclease with an 18 bp recognition sequence. In this protocol, transgenic *X. laevis* with knocked down expression of a specific gene are generated using a double promoter expression cassette. This cassette, which is flanked by I-SceI recognition sites, contains the shRNA of choice under the control of the human U6 promoter and a green fluorescent protein (GFP) reporter gene under the control of the human EF-1α promoter. Prior to microinjection the plasmid is linearized by digestion with I-SceI and the entire reaction is then microinjected into one-cell stage eggs. The highly stringent recognition sequence of I-SceI is thought to maintain the linearized plasmid in a nonconcatamerized state, which promotes random integration of the plasmid transgene in the genome. The injected embryos are reared until larval stage 56 and then screened for GFP expression by fluorescence microscopy and assessed for effective knockdown by quantitative RT-PCR using a tail biopsy. Typically, the I-SceI meganuclease transgenesis technique results in 35%–50% transgenesis efficiency, a high survival rate (>35%) and bright nonmosaic GFP expression. A key advantage of this technique is that the high efficiency and nonmosaic transgene expression permit the direct use of F_0 animals.

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 DNA extraction kit
Annealing buffer for shRNA (10×) <R>
Competent bacteria
DNA sequencing reagents
DNeasy Blood and Tissue Kit (QIAGEN) (optional; Step 15)

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From the *Xenopus* collection, edited by Hazel L. Sive.

Cite this protocol as *Cold Spring Harb Protoc*; doi:10.1101/pdb.prot101519

Ethanol (optional; see Step 10)
Ficoll (lyophilized powder, Type 400-DL)
Gentamicin (50 mg/mL)
GFP-specific PCR primers (optional; Step 15)
I-SceI-GFP-huU6-I-SceI plasmid (available upon request from the *Xenopus laevis* Research Resource for Immunobiology, https://www.urmc.rochester.edu/microbiology-immunology/xenopus-laevis.aspx)
I-SceI meganuclease (New England Biolabs R06943)
Luria Bertani (LB) ampicillin agar plates
Modified Barth's saline (MBS) <R>
PCR reagents for standard and qRT-PCR and target gene-specific primers
Phenol:chloroform:isoamyl alcohol (25:24:1) (optional; see Step 10)
Plasmid DNA isolation kits (mini and midi or maxi scale)
Restriction endonucleases appropriate for experimental design
shRNA oligonucleotides (designed according to Steps 1–2)
T4 DNA ligase
U6 promoter sequencing primer
Water (DNase/RNase-free) (optional; see Step 10)

Equipment

Agarose gel electrophoresis apparatus
Beaker (3 L minimum)
DNA sequencing apparatus
Fluorescence microscope
Heat block
Hot plate
Incubator
Microcentrifuge tubes (1.5 mL)
Microinjector (PLI-100, Harvard Apparatus or equivalent)
PCR apparatus (for standard and quantitative PCR)
Petri dishes (60 × 15 mm and 35 × 12 mm)
Scalpel (optional; Step 15)

METHOD

shRNA Design and I-SceI-GFP-huU6-shRNA-I-SceI Plasmid Preparation

1. Design shRNA oligonucleotides according to experimental goals. Include restriction sites at the 5′ ends of each oligonucleotide to facilitate shRNA cloning between the huU6 promoter and the I-SecI restriction site of I-SceI-GFP-huU6-I-SceI. (See Fig. 1 for potential restriction sites, which include BbsI, HindIII, ClaI, SalI, XhoI, and KpnI.) Use the following guidelines to design effective shRNAs.
    - Determine whether the gene of interest has one or multiple splice variants and decide whether to target all splice forms of the gene or specific variants. Select exons for targeting accordingly.
    - Design the shRNA so that the first nucleotide is a purine.
    - Choose a sequence with low (30%–50%) GC content.
    - Avoid the first 50–100 nt downstream from the start codon and the 100 nt upstream of the stop codon. Also avoid 5′- and 3′-UTRs. These regions contain binding sequences for regulatory proteins that may affect the accessibility of the RNA target sequence.

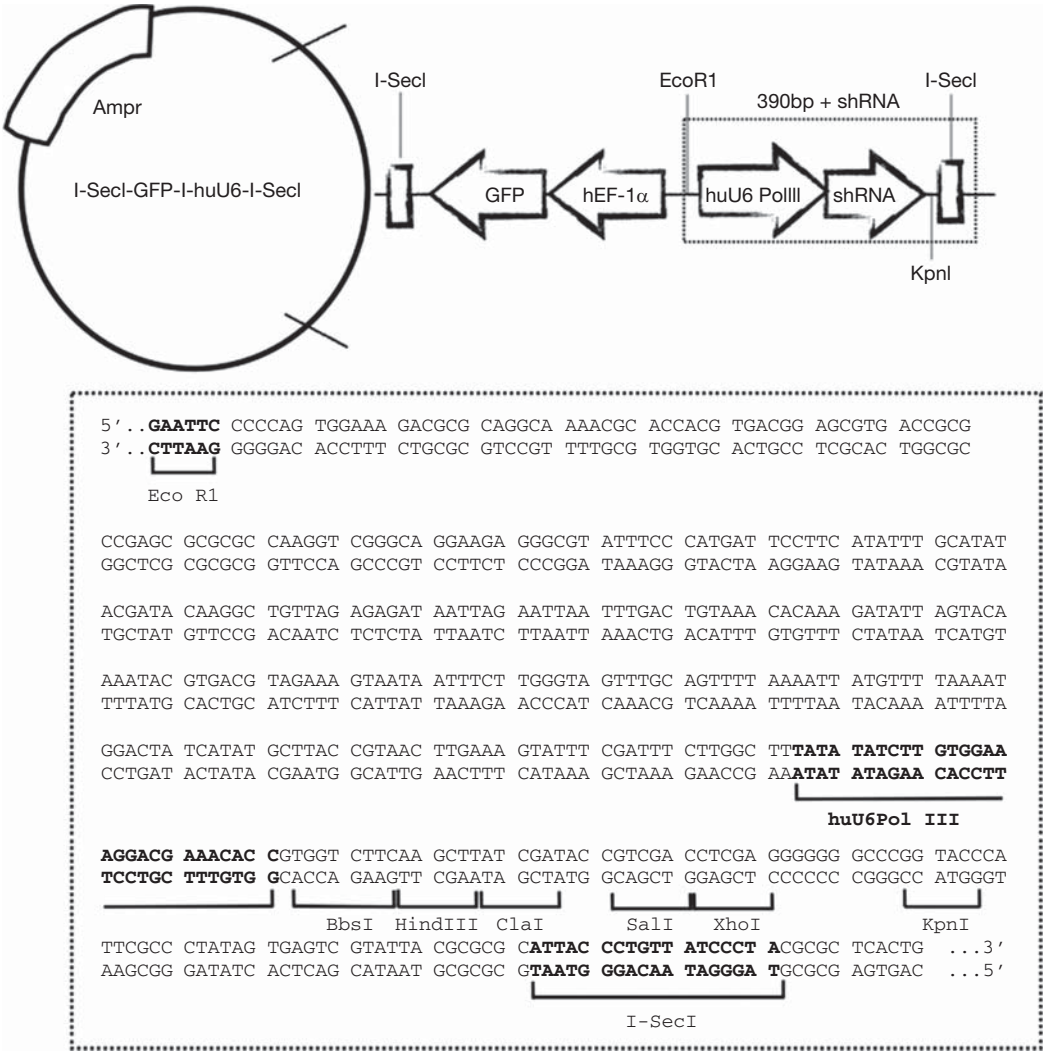

FIGURE 1. I-SceI-GFP-huU6-shRNA-I-SceI vector map and multiple-cloning site sequence. The sequence covering the shRNA cloning site includes the locations of potential restriction sites used to insert the shRNA into the vector.

- Design more than one shRNA per gene target.
- Ensure that the target sequence is specific for the gene of interest. Potential off targets can be identified by blast searching the shRNA sequence against the *X. laevis* genome available at http://www.xenbase.org/entry/.

There are several commercial and noncommercial websites available to assist in designing knockdown shRNAs and appropriate controls, including http://www.invivogen.com/sirnawizard/, http://biodev.extra.cea.fr/DSIR/DSIR.html, and http://www.genelink.com/sirna/shRNAi.asp.

shRNA oligonucleotide design is an empirical process and the gene silencing efficiency of each shRNA needs to be determined experimentally.

2. Design a control shRNA by randomly scrambling the target sequence.

   It is important to check the control shRNA for potential off target effects by blast searching the shRNA sequence against the X. laevis *genome.*

3. Synthesize or commercially obtain 40 nmol of each shRNA oligonucleotide in the 5′-phosphorylated form.
4. Resuspend 4 nmol of each shRNA in 10 µL 2× Annealing buffer and then combine the two oligonucleotides in a 1.5 mL microcentrifuge tube. Mix gently.

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5. Boil 2 L of water in a beaker. When water reaches boiling point turn off the hot plate and add the 1.5 mL microcentrifuge tube containing the oligonucleotides. Leave for a minimum of 6 h (or overnight) to allow oligonucleotides to anneal.
6. Double digest 1 µg of the I-SceI-GFP-huU6-I-SceI vector using restriction enzymes for the sites in the shRNA oligonucleotides. Digest for 1 h at 37°C. Heat inactivate digestion for 20 min at 65°C. Run the digest on a 1% agarose gel and perform gel purification of the digested vector using an agarose gel DNA extraction kit.
7. Ligate the annealed oligonucleotides from Step 5 into the digested I-SceI-GFP-huU6-I-SceI vector from Step 6 using T4 ligase. Combine 3.3 µL annealed shRNA, 3.3 µL digested I-SceI-GFP-huU6-I-SceI, 1 µL T4 ligase and 2 µL T4 10× buffer. Adjust volume to 20 µL with molecular grade H_2O. Ligate overnight at 16°C.
8. Transform 2.5 µL of the ligation reaction into competent bacteria using standard protocols. Select transformants on LB ampicillin agar plates.
9. Purify plasmid DNA from transformants using standard protocols and verify correct shRNA insertion by sequencing using U6 promoter primers.
10. Prepare plasmid DNA for digestion and microinjection using standard "midi- or maxi-prep" scale kits for isolation of pure plasmid DNA.

    Plasmid DNA can be further purified by phenol:chloroform:isoamyl alcohol (25:24:1) extraction, ethanol precipitation, and resuspension in DNase/RNase-free water.
11. Digest 1 µg I-SceI-GFP-huU6-shRNA-I-SceI with 10 units I-SceI meganuclease in a 25 µL volume for 40 min at 37°C.

    Store the I-SceI meganuclease at −80°C at all times (storage at −20°C leads to inefficient or no transgene incorporation into the genome). Freeze thawing of both the enzyme and the buffer significantly reduces transgenesis efficiency; therefore use single aliquots.

Microinjection of *Xenopus* Eggs

The microinjection of fertilized Xenopus *eggs is well documented and the precise methodology used will depend on the type of equipment available.*

12. Fertilize and dejelly eggs according to standard protocols and place eggs in a 35 × 12 mm injection Petri dish containing 0.3× MBS solution with 4% Ficoll. Ensure the eggs are completely submerged in the solution such that they are not subject to surface tension. Inject each egg with 10 nL of the digest from Step 11 containing 80 pg DNA and 1×10^{-3} U I-SceI.

    Use the I-SceI-GFP-huU6-shRNA-I-SceI digest within 15–30 min. If more eggs are to be injected, set up fresh digests. It is crucial that injection is performed within 30 min after fertilization and within 30 min after plasmid digestion to increase the likelihood of early integration. To avoid mosaicism it is advised that eggs are not injected once the first signs of cell division are observed.
13. Transfer injected eggs to a 60 × 15 mm Petri dish containing 0.3× MBS solution with 4% Ficoll and incubate for 4 h at 13°C.

    This delays cell division to extend the time for transgene integration.
14. Transfer the embryos to Petri dishes containing 0.3× MBS with 50 µg/mL gentamicin and rear the embryos at 18°C until hatching. Ensure that the embryos are completely submerged and not too crowded; between 50–100 embryos per dish is suitable.

    To ensure optimal survival of the embryos it is important to change the medium daily and to remove any dead embryos, which might otherwise compromise embryo survival.

    See Troubleshooting.
15. At developmental stage 58, screen tadpoles for GFP fluorescence using a fluorescence stereomicroscope.

    Alternatively, amputate the distal third of the tail with a scalpel and extract genomic DNA. Perform polymerase chain reaction (PCR) for the GFP transgene with GFP specific primers.

16. Select tadpoles with successful insertion of the GFP-containing vector and perform quantitative PCR to determine expression of the target gene. Verify target gene knockdown by comparing expression levels in shRNA-injected tadpoles with scrambled shRNA-injected age-matched controls.

See Troubleshooting.

TROUBLESHOOTING

Problem (Steps 14–15): Knockdown of a specific gene results in embryonic lethality.

Solution: Clone the shRNA of choice in a vector containing an H1 RNA polymerase III promoter repressed by the Tet-repressor. The expression of the shRNA can then be induced by inhibiting the tetracycline-element-specific repressor (TetR) from binding and blocking transcription with the tetracyclin analogue, doxycycline.

Problem (Step 16): Low to no knockdown of the gene of interest.

Solution: Design multiple shRNAs targeting the same gene and assess efficiency.

DISCUSSION

Different strategies to generate transgenic *Xenopus* frogs have been developed with specific molecular and technical features. The meganuclease approach is straightforward and results in high transgenesis efficiency with uniform expression of the transgene as well as stable germline transmission (Ogino et al. 2006a,b). One key advantage of this technique is that, because substantial numbers of nonmosaic embryos with high levels of knockdown are produced, it allows F_0 animals to be used for experimentation within a month after microinjection (Edholm et al. 2013). Another advantage of this knockdown technique is that different levels of silencing can be obtained in different animals, which may result in variable phenotypes. In addition, I-SceI meganuclease-mediated transgenesis has also been optimized for use with isogenic clones (LG 15 and LG 6 [Nedelkovska and Robert 2012; Nedelkovska et al. 2013]).

RELATED INFORMATION

We recommend Jensen et al. (2012) and Bofill-De Ros and Gu (2016) for in-depth discussion of shRNA design. These articles highlight important points to consider and potential pitfalls. In addition, see Ishibashi et al. (2012) for a detailed protocol describing I-SecI meganuclease-mediated transgenesis in *X. tropicalis*.

RECIPES

Annealing Buffer for shRNA (10×)

Reagent	Concentration
Tris-HCl (1 M, pH 7.5)	5 mL
NaCl (5 M)	10 mL
EDTA (0.5 M)	1 mL
H_2O	34 mL

Sterilize by autoclaving, and store at 4°C.

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Modified Barth's Saline (MBS)

$CaCl_2$ (0.1 M)
MBS salts (10×)

For a 1× solution of MBS, mix 100 mL of 10× MBS salts with 7 mL of 0.1 M $CaCl_2$. Adjust the volume up to 1 liter with H_2O. Store at room temperature.

MBS Salts (10×)

NaCl (880 mM)
KCl (10 mM)
$MgSO_4$ (10 mM)
HEPES (50 mM, pH 7.8)

Omit HEPES if MBS is to be used for oocyte maturation.

$NaHCO_3$ (25 mM)

Adjust pH to 7.8 with NaOH. Autoclave. Store at room temperature.

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Cold Spring Harb Protoc; doi: 10.1101/pdb.prot101519 originally published online January 30, 2018