Protocol

RNAi-Mediated Loss of Function of 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 *X* . This protocol describes the combination of transgenesis with stable RNA interference as an efficient reverse genetic approach to study gene function in *X* . Initially developed in the fish medaka and later adapted to *X* , this transgenic method uses the I-SceI meganuclease, a "rare-cutter" endonuclease with an 18 bp recognition sequence. In this protocol, transgenic *X. a* 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 ss ntial that you consult th appropriat Mat rial Sa ty Data Sh ts and your institution's Environm ntal H alth and Sa ty O fic or prop r handling o quipm nt and hazardous mat rials us d in this protocol.

RECIPES: Pl as s th nd o this protocol or r cip s indicat d by <R>. Additional r cip s can b ound onlin at http://cshprotocols.cshlp.org/sit /r cip s.

R ag nts

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)

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 *X* . *a* 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)

Equipm nt

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)

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-

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.

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 the known tetracycline-element-specific 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.

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)
O *B*
$NaHCO_3$ (25 mM)

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

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