Protocol

Raising Antibodies for Use in *Xenopus*

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For work in *Xenopus*, frog-specific antibodies must usually be raised, although a few antibodies against mammalian proteins cross-react. To produce an immunogen for antibody production, human embryonic kidney (HEK) expression systems can be used as described here. For most laboratories, the actual method of raising the antibody is determined by local ethical regulations controlling the adjuvant and injection protocols used. Because these steps are often outsourced, they are not included in this protocol.

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>.

The reagents and equipment needed depend entirely upon the choice of antigen and host. The materials listed here are specifically for HEK cell expression.

Reagents

Branched polyethylenimine (PEI; Sigma-Aldrich 408727)
ddH₂O
Dulbecco's modified eagle's medium (DMEM; Sigma-Aldrich D6546)
Ethanol (20%)
Fetal bovine serum (FBS)
Human embryonic kidney (HEK) 293T cells
Host animal and immunization reagents (see Step 5 and Steps 39–41)
JetPRIME reagent (Polyplus 114-01)
L-glutamine (100 mM)
Liquid nitrogen
M-PER™ Mammalian Protein Extraction Reagent (ThermoFisher Scientific 7850)
Nonessential amino acid solution (NEAAs; 100×; Sigma-Aldrich M7145)
Purification solution A <R>
Purification solution B <R>

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

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Cite this protocol as *Cold Spring Harb Protoc*; doi:10.1101/pdb.prot105585

Reagents for cloning, sequencing, transfection, western blotting, and cell culture (see Steps 6–16)

A comprehensive list of suitable plasmid expression vectors is provided by OPPF (<http://www.oppf.rc-harwell.ac.uk/OPPF/>).

Equipment

AKTA Start chromatography system (GE Healthcare Life Sciences)

BLAST program (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>)

Clustal Omega program (<http://www.clustal.org/omega/>)

Cryovials

Equipment for cloning, sequencing, transfection, western blotting, and cell culture (see Steps 6–16)

Filters (0.22 μ m)

Freezer (-80°C)

HisTrap FF column (1 mL; GE Healthcare Life Sciences, UK) or other appropriate column for the protein of interest

Incubator (37°C ; 5% CO_2 ; 95% air)

Protein structure prediction program (e.g., Phyre [<http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index>] or psipred [<http://bioinf.cs.ucl.ac.uk/psipred/>])

T175 flasks

Table top centrifuge

METHOD

The immunogenicity of the antigen depends on its own properties as much as on the choice of the host animal and the immunization protocol. To obtain the best result with antibody production, multiple approaches can be taken to produce the antigen and to purify the resulting serum, and the host can be varied (Bird et al. 2014; Delahaut 2017). All of these factors should be considered in order to obtain high-titer, high-affinity, and specific antisera.

Choosing the Best Target Sequence

When the full-length target protein cannot be generated in sufficient quantity or purity needed to immunize the chosen host, a peptide sequence can be used. To choose the most appropriate peptide sequence, three aspects have to be considered: its immunogenicity, the availability of the epitope in the protein's native form, and its uniqueness within the genome. Please note, some peptides might need to be coupled to a carrier protein produce a sufficient immune response (Trier et al. 2012).

1. BLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) the target protein sequence against the *Xenopus* proteome (six-way translated genome); reject regions that have high identities found in other proteins to avoid cross-reaction.
2. Determine the target region's secondary structure using prediction programs (e.g., Phyre [<http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index>] or psipred [<http://bioinf.cs.ucl.ac.uk/psipred/>]). Ensure that the protein's unique sequences are on the exposed protein surface.
These programs predict the probable spatial arrangement of the target sequence but are dependent on published knowledge about the sequence.
3. Run the final antigen sequence through BLAST once more to ensure there are no cross-reacting proteins.
4. Run a Clustal Omega (<http://www.clustal.org/omega/>) alignment for protein family members to ensure that BLAST did not miss any of these.

Choosing the Host Animal

5. Select an animal to be used as the host for antibody production (commonly used animals are listed below). Consider the final yield, cost, ethics, and ease of access to determine which one to use.

- Mouse (>6 wk old; 40 µg of <18 kDa antigen or 15 µg of >18 kDa; 50–70 µL preimmune bleed; 300 µL final bleed; animal culled)
- Rat (>6 wk old; 50 µg of <18 kDa antigen or 30 µg of >18 kDa; 1 mL preimmune bleed; 7–10 mL final bleed; animal culled)
- Guinea pig (>3 mo old; 50 µg of <18 kDa antigen or 30 µg of >18 kDa; 1 mL preimmune bleed; 7–10 mL final bleed; animal culled)
- Chicken and rabbit (>3 mo old; 200 µg of <18 kDa antigen or 100 µg of >18 kDa; 2 mL preimmune bleed; 50–70 mL final bleed; animal culled)
Polyclonal antibodies transmitted to chicken eggs are five to six times more concentrated than those in the blood but are difficult to extract and not widely used despite their advantages (Michael et al. 2010).
- Goat and sheep (6–7 mo old; 400 µg of <18 kDa antigen or 200 µg of >18 kDa; 2 mL preimmune bleed; 1 L final bleed; animal generally culled)
- Llama and horse (>18 mo–2 yr old; 400 µg of <18 kDa antigen or 200 µg of >18 kDa; 2 mL preimmune bleed; 1 L final bleed; animal can be reused)

Delahaut (2017) provides a concise and useful guide for immunizations.

Expressing the Immunogen

6. Clone the identified target sequence into a suitable vector, containing the appropriate promoter and C- or N-terminal fusion tags for enhanced expression and affinity purification.
7. Verify the sequence by Sanger sequencing.
8. Transfect HEK293T on a small scale using the JetPRIME reagent according to the manufacturer's instructions.
9. Confirm transfection efficiency by western blot and proceed with larger-scale expression.
10. Seed HEK293T cells at 7.5×10^5 cells/mL in 50 mL of complete DMEM and incubate in a T175 flask overnight at 37°C in a 5% CO₂, 95% air atmosphere.
11. When the cells reach 80% confluence, mix 87.5 µg of plasmid DNA with 2.6 mL of DMEM supplemented with 1× NEAAs and 1 mM L-glutamine at room temperature.
12. Separately, mix 154 µL of 1 mg/mL PEI with 2.6 mL of DMEM containing 1 mM L-glutamine at room temperature.
13. Mix the DNA-containing DMEM and the PEI-containing DMEM and incubate for 10 min at room temperature.
14. Remove the medium from the T175 flask and replace with 45 mL of fresh DMEM containing 2% FBS, 1× NEAAs, and 1 mM L-glutamine.
15. Incubate 10 min at room temperature.
16. Add the DNA/PEI solution to the cells and incubate at 37°C in a 5% CO₂, 95% air atmosphere for 3 d.

Proceed to Steps 17–20 for harvesting secreted proteins or Steps 21–23 for harvesting intracellular proteins.

Harvesting Expressed Proteins

Secreted Proteins

17. Collect the cell supernatant containing the protein.
18. Centrifuge at 6000g for 15 min at 4°C.

19. Filter the supernatant through a 0.22- μ m pore filter.
20. Store filtrate at 4°C.

Intracellular Proteins

21. To collect the cells from the medium, centrifuge at 6000g for 15 min at 4°C, and discard the supernatant.
22. Freeze the pelleted cells and the T175 flask containing the attached cells at –80°C.
23. Use M-PER™ Mammalian Protein Extraction Reagent or equivalent to lyse the cells and isolate the intracellular proteins according to the manufacturer's instructions.

Affinity-Purifying the Immunogen

Protein purification of tagged proteins via affinity chromatography can be performed in a wide variety of manners. Here we describe the use of HisTrap columns on an AKTA Start chromatography system. A similar protocol can be run manually, using gravity filtration, starting at Step 27.

All solutions that pass through the AKTA chromatography system must be filtered with a 0.22- μ m filter.

AKTA Start Set Up

24. Filter purification solutions A&B with a 0.22- μ m filter.
25. Set the AKTA Start on a “manual run” and flush with H₂O at a rate of 5 mL/min for 2 min.
26. Flush all lines with filtered H₂O.
27. Connect the column (here, a 1-mL HisTrap FF column), making sure no air bubbles are introduced.
28. Flush the column with five column volumes of purification solution A at <3 mL/min or at the rate specified by the column manufacturer.
29. Flush only the “elution line” with five column volumes of purification solution B at <3 mL/min or at the rate specified by the column manufacturer.
30. Prime the system by flushing with solution A, avoiding the “elution line.”

Sample Loading and Purification

31. Load the filtered sample, allowing it to flow through the column to bind.
32. Wash the column with at least five volumes of purification solution A at <3 mL/min or at the rate specified by the column manufacturer.
33. Set a gradient of 0%–100% solution B over 10 min.
34. Collect 1-mL fractions.

A peak on the UV absorbance shows elution of the protein.

35. Keep the flowthrough.

Cleaning and Storing the AKTA Start

36. Wash the column with purification solution A for 5 min and switch to solution B for 3 min.
37. Flush 20% ethanol through the AKTA Start and the column for 10 min.
38. Store the AKTA Start filled with 20% ethanol.

TABLE 1. Antibodies previously developed against *Xenopus* proteins

Target	Host	Type	Laboratory of origin	References	Availability ^a
Cell cycle components, focusing on protein kinases that regulate mitosis (cyclins, Cdc proteins, Atr, Bub, etc.)	Rabbit	Polyclonal	Maller laboratory	Gautier et al. 1988; Gautier et al. 1990; Maller 2012; Qian et al. 2001	EXRC
Cell cycle components (cyclin A, B1, B2, etc.)	Rabbit	Polyclonal	Hunt laboratory	Kobayashi et al. 1991; Minshull et al. 1990; Strausfeld et al. 1994	n/a
Key markers for the kidney, heart, nervous system, and muscle	Mice	Monoclonal	Jones laboratory	Balakrishna et al. 2011; Jones 2003; Lyons et al. 2009; Miller et al. 2011; Naylor et al. 2009; Simrick et al. 2005	EXRC
Cdc6, Orc2, Mcm3, etc.	Rabbit	Polyclonal	Various laboratories	Coleman et al. 1996	n/a
Uhrf1 and Dnmt1	Rabbit	Polyclonal	Lindsay laboratory	Taylor et al. 2013	n/a
Svv1, Sv2, Eiap, and Xiap	Rabbit	Unknown	Yamashita laboratory	Tsuchiya et al. 2005	n/a
Velo1	Goat	Polyclonal	Woodland laboratory	Nijjar and Woodland 2013	EXRC
Ilf3 and Nap1	Sheep	Polyclonal	Guille laboratory	Steer et al. 2003; Cazanove et al. 2008	EXRC

^aEXRC, European *Xenopus* Resource Centre.

Immunizing the Host

For most laboratories, the actual method for raising the antibody is determined by local ethical regulations controlling the adjuvant used and injection protocols. Often now these steps are outsourced, particularly as the preferred animal in which to raise polyclonal antibodies has changed from rabbits to sheep. For this reason, this protocol does not detail these steps.

39. (Optional) Consider coupling proteins <8–12 kDa to a carrier protein to enhance immunogenicity (Trier et al. 2012).

For octameric MAPs and long peptides, this is unnecessary.

40. Determine the need and type of adjuvant, bearing in mind that this is usually regulated by national and local rules (Delahaut 2017).
41. Arrange to immunize the host animal.

Generally, immunizations are carried out by companies or bioresource units with the appropriate licences in place.

Assessing the Immune Response

42. Proceed to Protocol: **Assessing the Immune Response When Raising Antibodies for Use in *Xenopus*** (Piccinni and Guille 2020).

DISCUSSION

To produce an immunogen, three expression systems are mainly used: in vitro cell-free extracts, *E. coli*, and HEK expression. In vitro expression depends solely on the manufacturer's instructions. These protocols can produce a wide range of soluble products but are costly and usually low yield, precluding production of immunogens for large animals (Delahaut 2017). Expression in *E. coli* produces large amounts of immunogen that have been successfully used for making several polyclonal *Xenopus*-specific antibodies (e.g., Steer et al. 2003; Cazanove et al. 2008). *E. coli*-produced proteins are poorly suited as antigens for monoclonal antibody production because contaminating *E. coli* proteins are likely to generate a stronger immune response than the desired *Xenopus* protein, resulting in the library of antibody-producing clones being dominated by those recognizing the *E. coli* contaminants (Joosten et al. 2003). More recently, expression in mammalian cells has been used to produce immunogens (e.g., Nettleship et al. 2015), and although more demanding and expensive than using *E. coli*, this method can produce sufficient protein for immunizing any host. Further, mam-

malian contaminants are not as immunogenic as those found in bacteria. As such, expression in HEK cells is described above.

Many antibodies, such as those in Table 1 and at <http://www.xenbase.org/reagents/antibody.do>, have already been developed specifically against *Xenopus* proteins and will be critical for understanding protein function and control in the *Xenopus* model. Some have been deposited and are available to the community through the European *Xenopus* Resource Centre (EXRC).

RECIPES

Purification Solution A

50 mM Tris-HCl, pH 7.5

500 mM NaCl

20 mM Imidazole

Store for up to 1 yr at room temperature.

Purification Solution B

50 mM Tris-HCl, pH 7.5

500 mM NaCl

500 mM Imidazole

Store for up to 1 yr at room temperature.

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Cold Spring Harbor Protocols

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Cold Spring Harb Protoc; doi: 10.1101/pdb.prot105585 originally published online January 3, 2020

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