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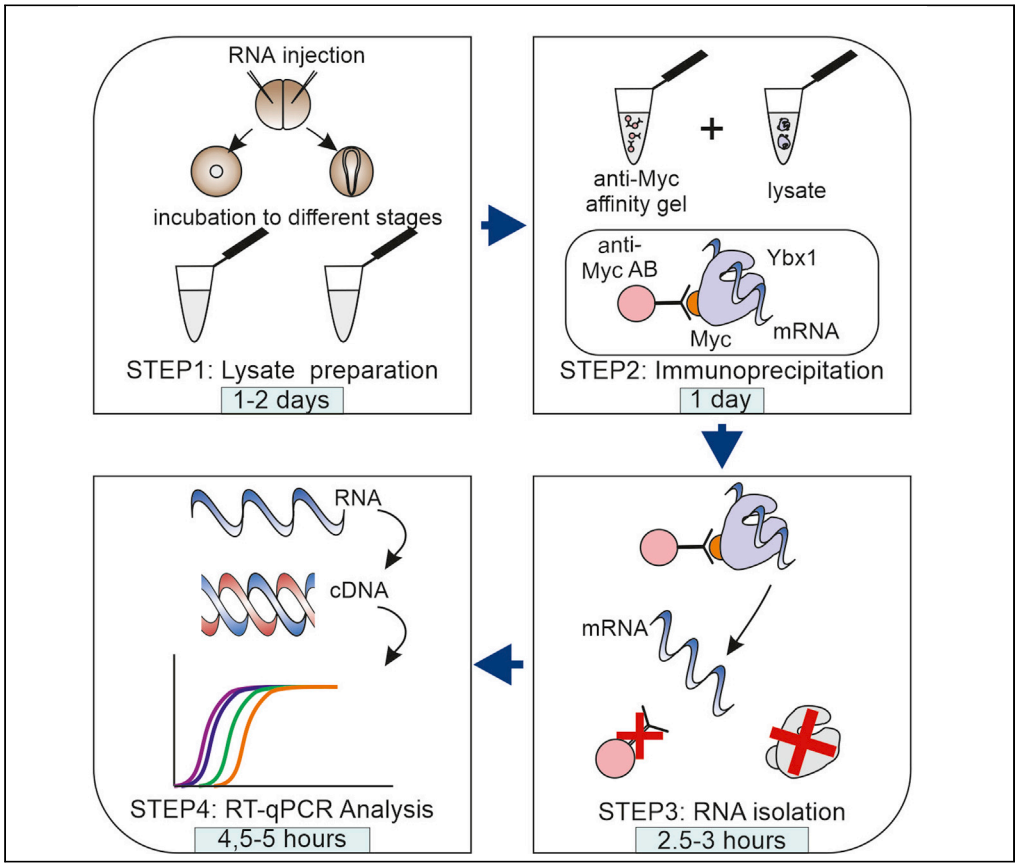
Protocol

Using RNA-binding proteins for immunoprecipitation of mRNAs from *Xenopus laevis* embryos



This protocol is developed for identifying mRNAs that form complexes with mRNA-binding proteins (mRBPs) in *Xenopus laevis* embryos at different developmental stages. Here, we describe the use of the Ybx1 mRBP for immunoprecipitation-based mRNA isolation. This protocol features the translation of the mRBP of interest directly in living embryos following injection of synthetic mRNA templates encoding a hybrid of this protein with a specific tag. This approach allows precipitation of mRNA-protein complexes from embryonic lysates using commercially available anti-tag antibodies.

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Highlights

Studying the repertoire of mRNA-binding proteins at different stages of development

Immunoprecipitation-based isolation of mRNAs from Xenopus laevis embryos

Precipitation of mRNA-protein complexes on commercially available carriers

Studying effects of the agents of interest upon the formation of mRNA-protein complexes

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Using RNA-binding proteins for immunoprecipitation of mRNAs from *Xenopus laevis* embryos

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SUMMARY

This protocol is developed for identifying mRNAs that form complexes with mRNA-binding proteins (mRBPs) in *Xenopus laevis* embryos at different developmental stages. Here, we describe the use of the Ybx1 mRBP for immunoprecipitation-based mRNA isolation. This protocol features the translation of the mRBP of interest directly in living embryos following injection of synthetic mRNA templates encoding a hybrid of this protein with a specific tag. This approach allows precipitation of mRNA-protein complexes from embryonic lysates using commercially available anti-tag antibodies.

For complete details on the use and execution of this protocol, please refer to Parshina et al. (2020).

BEFORE YOU BEGIN

mRNA-binding proteins play important roles in embryonic development, regulating expression at the posttranslational level. Optimized for use with the *Xenopus laevis (X.laevis)* embryo lysates, our mRNA immunoprecipitation protocol allows us to study the mRNA-binding repertoire of mRNA-binding proteins of interest at different stages of development. In this protocol, complexes consisting of a tagged mRNA-binding protein of interest, translated on the injected synthetic RNA templates encoding the same protein of interest, are formed with endogenous mRNAs in vivo in the cells of the developing embryo. The use of tagged exogenous protein enables the utilization of universal commercially available antibodies to precipitate the protein and avoid the nonspecific immunoprecipitation of structurally related proteins. In addition, this system allows additional agents (mRNA or anti-sense morpholino oligos) to be injected into the embryo to enhance or suppress the activities of other proteins, thereby revealing the effects of these proteins on the formation and stability of the ribonucleoprotein complexes of the mRNA-binding protein of interest.

Lysates of embryos have very high enzymatic activity; therefore, it is necessary to add inhibitors of proteinases and RNases immediately before use in accordance with the recommendations given in the protocol. In addition, tubes, separate pipettes and pipette tips, and RNase-free solutions prepared in Milli-Q water were used. As RNase and proteinase activity is known to decrease with temperature, it is recommended that the samples remain on ice and that centrifuges be set to 4°C.

The main steps are presented in Figure 1, left column, in blue.

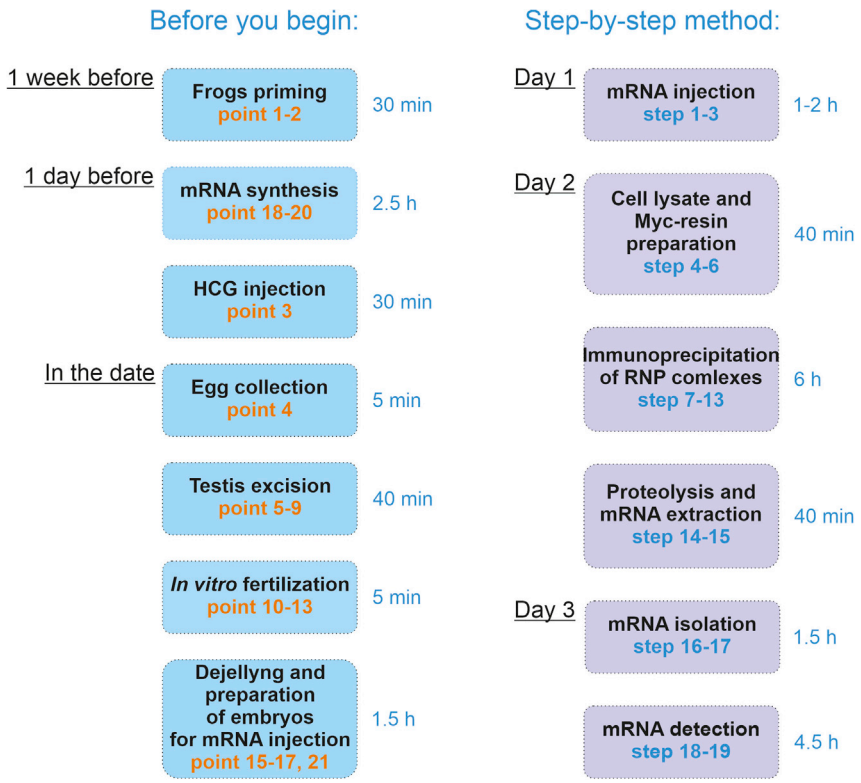

Figure 1. Overall schematic of workflow in this protocol
For each stage (dotted box), the expected finish time and the corresponding steps are annotated.

Inducing ovulation

Timing: 1 h for injections of Human Chorionic Gonadotrophin and frog handling (will depend on the number of frogs, we usually inject 3–4 female frogs), 1–3 days before micro-injection of synthetic RNA encoding tagged proteins of interest.

The protocol is based on Early Development of *Xenopus Laevis*: A Laboratory Manual (Sive et al., 2002).

1. Prepare stock solutions of Human Chorionic Gonadotrophin (HCG, 2,000 U/mL or 200 U/mL dissolved in water).
2. Prime *X.laevis* frogs by injecting them with 100–160 U of HCG into the dorsal lymph sac.
3. To induce ovulation, boost primed frogs by HCG injection. For *X.laevis*, we recommend injecting 1000–1600 U of HCG per frog 16–18 h before eggs are needed.

⚠ CRITICAL: The injected volume should not exceed 0.8 mL for *X.laevis*. Gently inject the hormones subcutaneously, dorsally posterior to the lateral line.

⚠ CRITICAL: Do not feed frogs for several days before ovulation induction. Feeding causes defecation during egg laying and affects egg quality.

Manual egg collection

Timing: 5 min

4. To obtain eggs, stroke the female on the back (Methods video S1), squeezing the eggs into a dry Petri dish. Leave the resulting eggs at 12°C until fertilization.) Eggs can be kept in the dry Petri dish without losing fertility for 1–1.5 h.

Isolation of testis

Timing: 40 min

5. Male *X.Laevis* must be put on ice until completely immobilized (see Methods video S2).

6. Cut out the testis (see Methods video S3).

7. Place the testis into a petri dish with 1× MMR.
8. Put 2 or 3 stitches with a silk suture needle (see Methods video S4).

9. Place the male in the tank water (Methods video S5). It can be used for fertilization one more time.

In vitro fertilization

Timing: 5 min

10. Cut a small piece (approximately 1/3) from the testis.
11. Rub each egg with this piece. Fertilize 1–2 clutches of eggs (several hundred eggs) with a cut piece (Methods video S6).

12. Pour 5 mL 0.1× MMR dropwise and incubate for 25 min at room temperature (22°C–24°C). (Methods video S7).

13. Fertilized eggs are turned upwards with an animal pole (see Methods video S8).

Dejellying embryos

Timing: 20 min

14. Dejelly embryos by removing buffer and swirling gently in 0.1× MMR with 2% (w/v) L-cysteine at pH 8.0. Dejellying is usually completed in 5 min. It is not necessary to change the Dejelly solution during this process (Methods video S8).
15. Rinse the fertilized eggs 5 times in 0.1× MMR (see Figure 2A).

16. Wait for the first cleavage (see Figure 2B). The start time of division depends on the temperature. Typically, at room temperature 22°C, division appears after 100 min. The start of division at a different temperature can be calculated from the development table available in Nieuwkoop and Faber, 1994.
17. Arrange the embryos in Terasaki plates with 4% ficoll in 0.1× MMR for microinjections (see Figure 2C).

⚠ CRITICAL: The second cleavage at room temperature occurs quickly, within 30 min, therefore it is better to carry out microinjections of synthetic mRNAs in a cool room at 16°C–18°C or use a Terasaki plates with ficoll cooled to 12°C. If a second division occurs, then you can continue injections into 4 blastomeres.

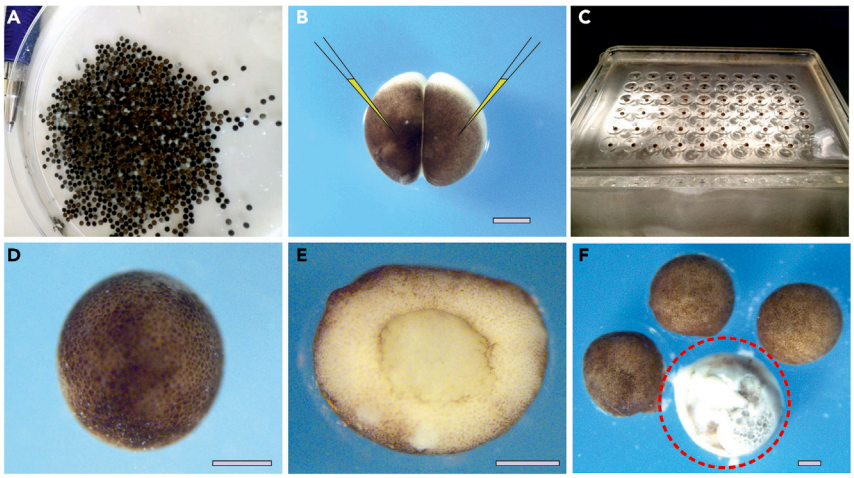

Figure 2. The main steps of embryo preparation (steps 15–17, before you begin and step 4, step-by-step method details)

(A) Fertilized eggs before cleavage begins.
(B) Two-cell stage embryo.
(C) Embryos in Terasaki plates before manipulation.
(D) Embryo at the gastrula stage (stage 12), top (animal) view.
(E) Embryo at the gastrula stage (stage 12), bottom (vegetal) view.
(F) Alive and dead (indicated by a red dashed line) embryos.
Scale bars are 500 μm.

Preparation of synthetic RNA encoding RNA-binding proteins of interest for microinjection into embryos

Timing: 2 days [Day 1 (one day before fertilization of eggs) - RNA synthesis and purification, day 2 – preparation of the microinjector and solutions for microinjection]

18. **Day 1.** Prepare synthetic mRNAs with mMessage mMachine SP6 or T7 Kit (Ambion) after linearization of plasmids.

⚠ CRITICAL: For the stability of synthetic RNA, we use vectors with poly-A sequences. To obtain Myc-tagged proteins, we used the *pCS2-MT* vector linearized with Not1.

Synthetic RNA must be capped to protect against degradation and stimulate translation; it is better to obtain from commercial kits containing a CAP analog in the nucleotide mixture.

19. Purify RNA by CleanRNA Standard kit.
20. Determine the concentration by Implen NanoPhotometer. Typically, 1 μg of purified RNA is required for one round of microinjections. Purified RNA can be stored at -20 C.

⚠ CRITICAL: After transcription and prior to embryo injection, it is essential to remove the cap analog, since it is a competitive inhibitor of cap-dependent translation. The CleanRNA Standard kit is sufficient to remove cap analog.

Note: Before performing the microinjections, it is important to assess the quality of the synthetic RNA. In this case, 1 μl of the obtained RNA is mixed with 0.3 μl of a dye (4× gel loading dye, blue, Evrogen) onto a 2% agarose gel and checked for the presence of the RNA band in a transilluminator in the presence of ethidium bromide. A clear band should be visible; A weak or low-molecular-weight band may indicate that the obtained RNA is of poor quality. If poor

RNA is suspected, it is highly recommended that test injections of the synthetic RNA to be used in the immunoprecipitation be injected into a small number of eggs to assess protein expression. The lysate obtained from 5–10 microinjected RNA eggs can be used to prepare a sample for Western blotting.

21. **Day 2.** Prepare instruments for microinjection:
    a. Pull nozzles backward in a low-flame gas burner (to fill microneedles (capillaries) for a microinjector, very fine nozzles are required. We use yellow pipette tips (200 µl), which we first soften on a gas burner with a small flame and quickly stretch to 20–25 cm (Methods video S9). After cooling, we cut the nozzles to 3 cm. You can also use the Eppendorf Microloader ™ (Cat # 5242956003)
    b. Prepare microneedles from glass capillaries using Narishige's PN-30 puller.
    c. Switch on the microinjector. Set injection parameters: p1 = 3400 hPa, p2 = 1150 hPa, p3 = 0.89 hPa, time = 0.1 s. These parameters can be used for Microinjector FemtoJet 4× (Eppendorf, see key resources table).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse monoclonal anti-c-Myc, AP-conjugated 1:1000 (for WB)	Sigma-Aldrich	Cat#A5963; RRID:AB_258265
Anti-rabbit IgG, AP-conjugated, produced in goat 1:30000 (for WB)	Sigma-Aldrich	Cat#A3937; RRID:AB_258122
Mouse monoclonal anti-alpha-Tubulin 1:1000 (for WB)	Sigma-Aldrich	Cat#T9026; RRID:AB_477593
Anti-mouse IgG, AP-conjugated 1:30000 (for WB)	Sigma-Aldrich	Cat#A3562; RRID:AB_258091
EZview Red Anti-c-Myc Affinity Gel	Sigma-Aldrich	Cat#E6654; RRID:AB_10093201
EZview Red ANTI-FLAG M2 Affinity Gel	Sigma-Aldrich	Cat#F2426; RRID:AB_2616449
Chemicals, peptides, and recombinant proteins		
Human chorionic gonadotropin	Sigma-Aldrich	Cat#CG10
NotI	Fermentas	Cat#ER0592
Western Blue® Stabilized Substrate for Alkaline Phosphatase	Promega	Cat#S3841
Sodium chloride (NaCl)	Helicon	Cat#H-1401
Potassium chloride (KCl)	Helicon	Cat#H-1101
Calcium chloride dihydrate ($CaCl_2$*$2H_2O$)	AMRESCO	Cat#0556
Magnesium chloride hexahydrate ($MgCl_2$*$6H_2O$)	Panreac	Cat#141396
HEPES	Panreac	Cat#A3724
L-Cysteine	Dia-m	Cat#M52904
Ficoll	Dia-m	Cat#PS400
Sodium hydroxide (NaOH)	Dia-m	Cat#145881
Ethanol	N/A	N/A
Isopropanol	N/A	N/A
Nonidet P-40	Helicon	Cat# Am-E109
DTT	Fermentas	Cat#RO861
RNase Out RNase inhibitor, 100 units/mL	Invitrogen	Cat#10777-019
Vanadyl ribonucleoside complexes (VRC)	New England Biolabs	Cat#S1402S
Protease Inhibitor Cocktail	Sigma-Aldrich	Cat#P8340
Tris	Helicon	Cat#Am-O497
EDTA	Helicon	Cat#Am-O105B
Glycerol	Helicon	Cat#I-800687
Sodium dodecyl sulfate (SDS) powder	Sigma-Aldrich	Cat# L4509
Beta-mercaptoethanol	Sigma-Aldrich	Cat#M6250

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Proteinase K, recombinant, PCR Grade	Roche	Cat#3115836001
Fluorescein Lysine Dextran (FLD) 40 kD	Invitrogen	Cat#D1845
Critical commercial assays		
mMessage mMachine™ SP6kit	Thermo Fisher	Cat#AM1340
ExtractRNA	Evrogen	Cat# BC032
CleanRNA Standard	Evrogen	Cat# BC033
MMLV RT kit	Evrogen	Cat# SK021
qPCRmix-HS SYBR	Evrogen	Cat# PK147L
Experimental models: organisms/strains		
Wild-type *Xenopus laevis* frog males and females aged 1-3 years	Nasco	Cat#LM00456; RRID:XEP_Xla100
Recombinant DNA		
pCS2MT-Ybx1, the plasmid is available upon request	Parshina et al., 2020	N/A
Software and algorithms		
Excel	Microsoft	N/A
Other		
2 mL Syringe with 23G×1¼" needle	Medical Products	Cat#V160218
Mini-centrifuge-vortex Microspin	BioSan	Cat#FV-2400
Flake Ice Machine	Porkka	Cat#KF45
Surgical scissors	N/A	N/A
Forceps	N/A	N/A
Magnetic Glass Microelectrode Horizontal Puller	Narishige	Cat#PN-30
Microcentrifuge	Eppendorf	Cat#5415
Thermostat "Gnom"	DNA-Technology	N/A
Microcentrifuge MiniSpin	Eppendorf	Cat#5452 000
Implen NanoPhotometer C	Implen	Cat# C40*
Leica modular systems	Leica	Cat#AF6000
Pipetmans	Gilson	N/A
Nutator	Clay Adams	Cat#421105
Microinjector FemtoJet 4×	Eppendorf	Cat# 5253000017
DTprime Real-time Detection Thermal Cycler	DNA-Technology	N/A
Petri dish	Greiner	Cat#628102
Terasaki plate	Greiner	Cat#659190
Serum pipette	Greiner	Cat#612301
Glass capillaries with one (or two) filament 1.2 mm	SIMAX	N/A
Microcentrifuge tubes	SSI	Cat#1260-00
Pipette tips	SSI	N/A
SnapStrip PCR Tubes with Caps	SSI	Cat#3245-00

MATERIALS AND EQUIPMENT

Solutions used for obtaining embryos

20× MMR		
Reagent	Final Concentration	Amount
NaCl	2 M	58.45 *g*
KCl	0.04 M	1.43 *g*
$CaCl_2$*$2H_2O$	0.04 M	2.94 *g*
$MgCl_2$*$6H_2O$	0.02 M	2.03 *g*
Milli-Q water	To a final volume of 500 mL	
Total	**n/a**	**500 mL**
Can be stored at +4°C for several months		

200× Hepes		
Reagent	Final Concentration	Amount
Hepes	1 M	119.15 *g*
Milli-Q water	To a final volume of 500 mL	
Total	1 M	500 mL
Adjust pH to 7.4 with NaOH. Can be stored at +4°C for several months		

1× MMR		
Reagent	Final Concentration	Amount
20× MMR	N/A	5 mL
200× Hepes	5 mM	0.5 mL
ddH_2O	To a final volume of 100 mL	
Total	n/a	100 mL
Can be stored at +4°C for several months		

0,1× MMR		
Reagent	Final Concentration	Amount
20× MMR	N/A	5 mL
200× Hepes	5 mM	5 mL
ddH_2O	To a final volume of 1000 mL	
Total	n/a	1000 mL
Can be stored at room temperature for several months		

L-Cysteine		
Reagent	Final Concentration	Amount
L-Cysteine	2 % (w/v)	2 *g*
0,1× MMR	To a final volume of 100 mL	
Total	n/a	100 mL
Adjust pH to 7.8 with NaOH. Prepare on the day of microinjection		

Ficoll		
Reagent	Final Concentration	Amount
Ficoll	4% (w/v)	4 *g*
0,1× MMR	To a final volume of 100 mL	
Total	n/a	100 mL
Can be stored at +4°C for a month		

NaOH		
Reagent	Final Concentration	Amount
NaOH	1 M	20 *g*
Milli-Q water	To a final volume of 50 mL	
Total	1 M	50 mL
Can be stored at room temperature for several months		

Solutions used for injection of synthetic mRNA

FLD		
Reagent	Final Concentration	Amount
Fluorescein Lysine Dextran	50 μg/μL	25 mg
Milli-Q water	n/a	500 μL
Total	50 μg/μL	500 μL
Can be stored at -20°C for several years		

Solutions used for immunoprecipitation of ribonucleoprotein complexes

KCl		
Reagent	Final Concentration	Amount
KCl	1 M	3.72 *g*
Milli-Q water	To a final volume of 50 mL	
Total	1 M	50 mL
Can be stored at room temperature for several years		

$MgCl_2$		
Reagent	Final Concentration	Amount
$MgCl_2*6H_2O$	2 M	20.33 *g*
Milli-Q water	To a final volume of 50 mL	
Total	2 M	50 mL
Can be stored at +4°C for several years		

NaCl		
Reagent	Final Concentration	Amount
NaCl	5 M	14.61 *g*
Milli-Q water	To a final volume of 50 mL	
Total	5 M	50 mL
Can be stored at +4°C for several years		

DTT		
Reagent	Final Concentration	Amount
DTT	1 M	1.54 *g*
Milli-Q water	To a final volume of 10 mL	
Total	1 M	10 mL
Can be stored 1 mL aliquots at -20°C for several years		

Tris-HCl		
Reagent	Final Concentration	Amount
Tris	1 M	6.05 *g*
Milli-Q water	To a final volume of 50 mL	
Total	1 M	50 mL

1 Adjust pH to 6.5 with HCl for Sample buffer.
2 Adjust pH to 7.4 with HCl for NT2 buffer.

Can be stored at +4°C for several years

EDTA		
Reagent	Final Concentration	Amount
EDTA	500 mM	9.31 *g*
Milli-Q water	To a final volume of 50 mL	
Total	500 mM	**50 mL**
Adjust pH to 8.0 with NaOH. Can be stored at +4°C for several years		

Buffers used for immunoprecipitation of ribonucleoprotein complexes

Polysome lysis buffer		
Reagent	Final Concentration	Amount
KCl (1 M)	100 mM	100 µL
$MgCl_2$ (2 M)	5 mM	2,5 µL
200× Hepes	10 mM	10 µL
Nonidet P-40	0.5% (v/v)	5 µL
DTT (1 M)	1 mM	10 µL
RNAse Out	100 U/mL	2.5 µL
VRC (200 mM)	400 µM	2 µL
Protease Inhibitor Cocktail	4% (v/v)	200 µL
Milli-Q water	n/a	668 µL
Total	**n/a**	**1 mL**
Must be prepared on the day of the experiment		

NT2 buffer		
Reagent	Final Concentration	Amount
Tris-HCl (1M, pH 7.4)	50 mM	2.5 mL
NaCl (5 M)	150 mM	1.5 mL
$MgCl_2$ (2 M)	1 mM	25 µL
Nonidet P-40	0.05 % (v/v)	25 µL
Milli-Q water	To a final volume of 50 mL	
Total	**n/a**	**50 mL**
Must be prepared on the day of the experiment		

Sample buffer for western blot

4× Laemmli buffer		
Reagent	Final Concentration	Amount
Glycerol	10% (v/v)	1 mL
Tris-HCl (1M, pH 6.5)	125 mM	1.25 mL
SDS	8% (w/v)	0.8 *g*
Beta-Mercaptoethanol	5 % (v/v)	500 µL
Milli-Q water	To a final volume of 10 mL	
Total	**n/a**	**10 mL**
Can be stored 1 mL aliquots at -20°C for several years		

Experimental model and subject details

Mature *X. laevis* frogs were obtained from NASCO (Fort Atkinson, WI). *X.laevis* frogs were maintained in a recirculating tank system with regularly monitored temperature and water quality. *X. laevis* were housed at a temperature of 18°C. All experimental protocols involving frogs were

performed in accordance with guidelines approved by the Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry (Moscow, Russia) Animal Committee and handled in accordance with the Animals (Scientific Procedures) Act 1986 and Helsinki Declaration.

STEP-BY-STEP METHOD DETAILS

The main steps are presented in Figure 1, right column, in purple.

Injection of synthetic mRNA encoding RNA-binding proteins of interest into embryos

Timing: 1–2 h

1. **Preparation of microinjection mixture.** We used a concentration of 70 ng/μL *myc-ybx1* RNA mixed with vital FLD dye (the vital fluorescein lysine dextran dye is used to control the microinjected material in the embryos. It does not affect the development of the embryos or the translation of the injected RNA (Sive et al.,2002)).

Typically, the total volume of the microinjection mixture is 10 μL. RNA is diluted on the basis of its concentration after purification, and 0.5 μl of FLD per mixture is added from a standard 50 μg/μL solution (see Solutions used for injection of synthetic mRNA).

RNA and FLD is mixed immediately before injections, and all manipulations are performed on ice. The resulting mixtures must be centrifuged for 5 min at 13400 *g* at 4°C to prevent capillary clogging.

> *Note:* Tracer FLD is distributed more evenly in relation to mRNA. mRNA diffuses slowly from the injection site, and during cell division, the distribution can be uneven. To track the post-injection mRNA pathway more accurately, clone indicators such as green fluorescent protein (GFP) mRNA can be introduced together with the tested mRNA. Although the injection of indicator mRNA is useful, it is known to compete for translation with coinjected test RNA (K. Kintner, personal communication). In studies using mRNA microinjections of fluorescent proteins, we observed a visually uniform and extensive distribution of fluorescent protein products in embryos at late stages (Matz et al., 1999)

2. Fill the capillary with 10 μL of the mRNA-FLD mixture using the drawn-out tips.

The capillary is placed into the microinjector and broken off with a light touch (see Figures 3A–3C).

The injection volume is calibrated by allowing the drop to hang at the end of the needle, where its diameter can be measured using an eyepiece micrometer and calculate the injected volume ($v = 4/3\ \pi\ r^3$, where v is the volume and r is the radius of the drop, or Table 8.1 in Sive et al., 2002 can be used).

The prepared embryos are injected into any area of both blastomeres (see Figure 2B).

> *Note:* The translation efficiency of the synthetic mRNA in embryos is highly variable between RNAs. Usually, mRNAs obtained as a result of transcription of the linearized *pCS2MT* vector provide a high level of protein expression in embryonic cells — up to 100 ng of protein per embryo (Martynova et al., 2008). After RNA purification with a CleanRNA Standard kit, a concentration of approximately 400 ng/μL is usually obtained. This concentration of the obtained RNA does not require additional concentration.

In the case of an insufficient concentration, precipitation of the RNA with a 70% ethanol solution containing 150 mM NaCl (incubated 2–24 h at −20°C) can be used, followed by washing with 96%

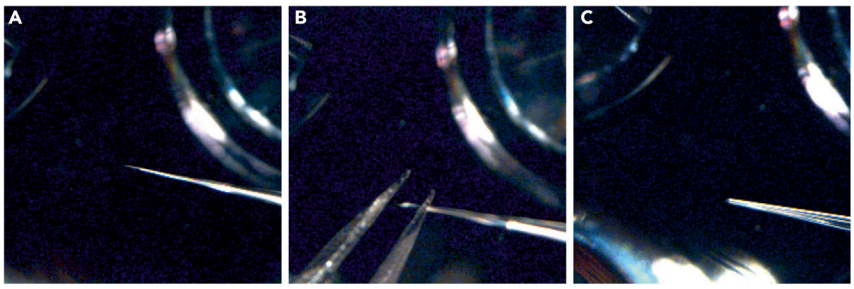

Figure 3. The main steps of capillary preparation (step 2, step-by-step method details)
(A) Initial capillary
(B) The capillary is broken off with a light touch.
(C). Prepared capillary after tip break

ethanol, drying the resulting RNA precipitate and dissolving in it a small volume of RNAse-free water. The resulting RNA can be stored at −70°C for several months.

⚠ CRITICAL: Diluted RNA may degrade rapidly; therefore, it is best to reprecipitate it for long-term storage. In addition, to avoid the effects of thawing-freezing cycles, it is advisable to store it in small aliquots (10 μL) at −70°C.

When working with RNA, separate pipettes, tips, and RNA-free water are used. To avoid the ingress of RNA-destroying enzymes during handling, rubber gloves are worn, preferably in a workplace specially designated for RNA extraction.

3. Incubate the embryos in 4% Ficoll for 1–2 h and then place them in Petri dishes with 0.1× MMR (Solutions used for obtaining embryos) and incubate until the desired stage.

Note: Working with *X. Laevis.* embryos is convenient because their development can be controlled by temperature. Using the development table (Nieuwkoop and Faber, 1994), the incubation time can be adjusted by changing the temperature. Usually, at room temperature (18°C–20°C), embryos injected during the day reach the gastrula stage by the morning of the next day.

Preparation of lysate from embryonic cells

Timing: 45 min

4. Collect embryos at the research stage (we work at the stage of middle gastrula, stage 11 according to the development table, Nieuwkoop table, see Figures 2D and 2E and Martynova et al., 2021, STAR Protocols: "The protocol for separation of the nuclear and the cytoplasmic fractions of Xenopus laevis embryonic cells for studying protein shuttling"). Place the embryos in Eppendorf tubes on ice, carefully remove the water, and add polysome lysis buffer (Buffers used for Immunoprecipitation of Ribonucleoprotein Complexes) with protease and RNase inhibitors at a rate of 10 μl of buffer per 1 embryo. We use 30 embryos at each point of the experiment (for each injection mixture). Embryos should be broken up by pipetting up and down several times. The mRNP (messenger ribonucleoprotein) lysate is allowed to incubate on ice for 5 min and is centrifuged for 30 min at 18000 *g*. Collect the supernatant carefully. (The bold layer on top can be collected if it is mixed with the supernatant; its inclusion will not affect the following steps.)
5. We use 300 μl of mRNP lysate for each injection mixture. The lysates can be frozen (flash-freeze) and stored at −70°C, but freezing can affect the protein conformation and reduce the stability of the RNP complexes. Therefore, we use only fresh lysates for RNA precipitation. Five to ten microliters of lysate is added to an equal volume of 4× Laemmli buffer (Sample buffer for Western blot)

for use in the Western blot analysis of tagged protein expression. The samples are boiled for 10 min at 80°C and stored at −20°C until use. Samples in Laemmli buffer can be stored for several months.

⚠ CRITICAL: It is important to collect live embryos containing the microinjected material. These can be detected by FLD fluorescence under a fluorescent microscope with a GFP filter. Dead embryos (easily visible as white spheres without dividing cells) non-FLD embryos are discarded (see Figure 2F and Martynova et al., 2021, STAR Protocols: "The protocol for separation of the nuclear and the cytoplasmic fractions of Xenopus laevis embryonic cells for studying protein shuttling").

Preparation of Myc-resin

Timing: 30–45 min

6. We use a commercial agarose-based carrier with immobilized antibodies (key resources table, Sigma, E6654 EZview Red Anti-c-Myc Affinity Gel). Each point of the experiment requires 50 μL of affinity gel.
   a. For affinity gel blocking, add 250 μl of EZview Red Anti-c-Myc Affinity beads (used at 5 points in the experiment) to 1.5 mL of NT2 buffer that contains 5% BSA (in a 2-mL Eppendorf tube) and incubate for 10–30 min on a rotator at 20°C–22°C .
   b. To wash the affinity gel, add 1.5 mL of NT2 buffer, mix it by inversion and centrifuge it at 2500 *g*.
   c. Wash the gel 2–3 times.
   d. After washing, equilibrate the resin with 0.5 mL of the NT2 buffer with protease and RNase inhibitors (Buffers used for Immunoprecipitation of Ribonucleoprotein Complexes).

⚠ CRITICAL: To determine the amount of RNA nonspecifically bound to the affinity gel, we use a commercial FLAG gel — EZview Red ANTI-FLAG M2 Affinity Gel (key resources table, F 2426, Sigma) with antibodies against the FLAG peptide, which is not used as a tag in our experiments. The amount of gel used and the method of its preparation do not differ by experiment.

Note: A major disadvantage of affinity-based molecular pull-down and immunoprecipitation procedures is that the affinity matrix is difficult to see in the microcentrifuge tube following centrifugation steps.

We used EZview ™ Red Affinity dye conjugated agarose (Sigma). The vivid red color of the affinity beads provides high visibility that allows easy differentiation of the pellet from the supernatant, therefore reducing the risk of accidental aspiration of the pellet and allowing for less tedious manipulations.

Immunoprecipitation of Myc-tagged ribonucleoprotein complexes

Timing: 6 h

7. Divide the washed and buffered resin by the number of samples into equal volumes. Centrifuge for 3 min at 2500 *g*, remove the supernatant.

⚠ CRITICAL: Do not centrifuge the Affinity Gel at high speed (not higher than 3 000 *g*), it may break down.

8. Dilute lysate and mixing with affinity gel

a. Resuspend Anti-c-Myc Affinity beads in 850 μL of ice-cold NT2 buffer. Add 200 units of an RNase inhibitor (5 μL RNase Out), 2 μL (to final concentration of 400 μM) Vanadyl ribonucleoside complexes, 10 μL of 100 mM DTT and EDTA to 20 mM and 2 μL Protease Inhibitor Cocktail (Solutions used for Immunoprecipitation of Ribonucleoprotein Complexes).
b. Thaw mRNP lysate on ice and centrifuge at 15 000 *g* for 15 min to clear lysate of large particles. Transfer cleared supernatant to microfuge tube and store on ice.
c. Add 100 μL of cleared lysate to bead mixture prepared in step 8a.

⚠ CRITICAL: This dilution of lysate is important to reduce unspecific binding (dilution of lysate performed according to Keene et al., 2006).

9. Immediately flick tube several times with a finger to mix, and centrifuge briefly (within 15 s) at 3000 *g* to pellet beads.
10. To represent total cellular mRNA, remove 100 μL of supernatant, and add 1 mL of ExtractRNA to the sample, mix well by vortexing. Maintain at −20°C until use in step 16.

Note: Since the amount of mRNA coprecipitated with the antibody is calculated as a percentage of the total input, this volume (approximately 11% of the total volume) is required to determine the total amount of mRNA at each point in the experiment and is taken directly from the tube where binding will take place before incubation.

11. Incubate the mixture for 4 h at 4°C while tumbling end over end on a rotator at low speed.
12. Pellet beads by centrifugation at 3000 *g* for 5 min and save supernatant for later analysis if desired. Supernatant may be stored at –20°C for several months.
13. Wash beads 4–5 times with 1 mL of ice-cold NT2 buffer by centrifugation at 3000 *g* for 5 min and removing supernatant with a hand pipettor or an aspirator.

⚠ CRITICAL: Thorough washing is critical for reducing background. All tubes should be kept on ice as much as possible while working quickly during the washing process to reduce degradation.

Proteolysis and RNA isolation

Timing: 40 min

14. After the final wash, resuspend the beads in 100 μL of NT2 buffer. Proteinase K (30 μg per 100 μL of NT2 buffer) is added to release the RNP components. The mixture is incubated for 30 min at 55°C, the tube flicked occasionally with a finger.

Note: To validate the immunoprecipitation results by Western blotting, 10 μL of the immune-bound complexes are mixed with 10 μL of 4× Laemmli buffer (Sample buffer for Western blot) and boiled for 10 min at 80°C. These complexes are stored at −20°C until use.

15. Release the RNP components and isolate the RNA from the immunoprecipitated pellet by adding 1 mL of ExtractRNA to the remaining 100 μL of the beads containing the protein-RNA immunocomplexes. The beads in solution are mixed well by vortexing 15 s to allow the elution of the RNA from the protein-antibody-bead complexes.

Note: The sample volume should not exceed 10% of the volume of the ExtractRNA used for lysis.

Extraction of mRNA

Timing: 1.5 h

16. Isolate RNA using ExtractRNA:
    a. Incubate samples at room temperature (22°C–24°C) for about 15 min.
    b. Centrifuge at maximum speed (12 000–15 000 *g*) at room temperature for 10 min.
    c. Take the supernatant into a new tube and add 0.2 mL of chloroform to it. Mix vigorously by inverting for 15 s.
    d. Incubate at room temperature for 2–3 min.
    e. Centrifuge samples at 12 000 *g* at + 4°C for 15 min.
    f. During centrifugation, the liquid is separated into three phases, take the upper aqueous phase containing RNA into a new tube and add 500 µl of isopropanol to it. Mix well by vortexing.
    g. Incubate the mixture for 10 min at room temperature.
    h. Centrifuge samples at 12000 *g* for 10 min at room temperature. Remove the supernatant carefully.
    i. Add 1 mL of 75% ethyl alcohol to the sediment along the wall of the test tube.
    j. Centrifuge at maximum speed (12 000–15 000 *g*) for 5 min at room temperature.
    k. Remove supernatant
    l. Air dry the RNA precipitate.

    ⚠ CRITICAL: The RNA precipitate is allowed to dry for no more than 10 min. When working with RNA, it is important to prevent the precipitate from air-drying for a long time period, as this can negatively impact RNA solubilization.
    m. Add 100 µl of RNase-free water to the dried RNA precipitate

17. Purify RNA using CleanRNA Standard kit:
    a. Add 350 µL of RNA Binding Solution (a part of the kit) to the sample. Vortex.
    b. Add 250 µl 96% ethanol. Mix by inverting the tube.
    c. Transfer the sample to the spin column.
    d. Centrifuge for 30 s at maximum speed (12 000–15 000 *g*) to adsorb RNA on the column filter.
    e. Wash RNA with RNA Wash Solution (a part of the kit) twice. Centrifuge the empty column for 5 min to completely dry the column filter.
    f. Wash off RNA with **10–15** µl of RNase-free water heated to + 50°C (for better separation of RNA from the column).

    Note: Since there is little RNA, it is better to elute with the minimum possible volume of RNase-free water (10–15 µl)
    g. RNA concentration and quality can be assessed by an Implen NanoPhotometer. Usually, the concentration to use is approximately 100 ng/µl.

RNA detection by RT-qPCR

Timing: 4.5 h

18. Prepare cDNA from the coIPed RNA sample and quantify the specific transcript abundance by real-time quantitative PCR (RT-qPCR).
    a. Perform the reverse transcription reaction with the MMLV RT kit. For the reverse transcription reaction, use 200 ng RNA template. Prepare mixture I in a sterile tube.

Mixture I	Volume
RNA (100 ng/µl)	6 µl
Oligo-dT primers (20 µM)	1.5 µl
Random primers (20 µM)	1.5 µl
Total	9 µl

Warm up the mixture for 2 min at 70°C and transfer the samples to ice. Add 10 μl of the pre-mixed mixture II.

Mixture II	Volume
RNAse free water	2 μl
5× first strand synthesis buffer	4 μl
dNTP Mix (10 mM each)	2 μl
DTT	2 μl
Total	**10 μl**

Add 1 μl of MMLV reverse transcriptase to the test tubes. Incubate the mixture for 45 min at 37°C.

To inactivate the enzyme, heat the mixture for 10 min at 70°C.

Pause point: cDNA can be stored at −20°C until use.

b. To carry out the PCR reaction, use the ready-made qPCRmix mixture in accordance with the manufacturer's instructions.

Mixture for qRT-PCR	Volume
Milli-Q water	14 μl
5× reaction mixture qPCRmix-HS SYBR	5 μl
Specific primers (1,25 mM each)	2.5 + 2.5μl
cDNA	1 μl
Total	**25 μl**

19. Introduce the plate in the Thermal Cycler and set a program (an example is shown in Table PCR reaction protocol).

PCR Cycling Conditions			
Steps	Temperature	Time	Cycles
Initial Denaturation	92°C	1 min	1
Denaturation	94.5°C	15 s	45
Annealing + Extension	60°C	25 s	

Note: RNA target enrichment among the RBP-interactome can be calculated from RT-qPCR data following the pipeline explained in Quantification and Statistical Analysis section.

EXPECTED OUTCOMES

Due to the use of a specific tag (Myc, FLAG or another tag) and the commercially available antibodies for the same tag, this protocol allows, first, efficient selection of pools of mRNA that specifically bind in embryonic cells to any mRNA-binding protein of interest and, second, minimization of the precipitation of structurally similar proteins. In our work, we use elution with ExtractRNA, but in the case of low specificity, the purity of the protein can be obtained by eluting specific RNP complexes with Myc-peptide. In addition, incubation of the lysates with Myc-binding resin in the presence of Myc-peptide can be used as a negative control. Moreover, proteins with Myc-tags that do not interact with RNA or a mutant of the protein of interest without an mRNA-binding domain can also be used for this purpose. Thus, in our work, for the negative control, we use a mutant of

Ybx1 with C-terminal deletion and a Myc-tag. Mutant Ybx1 lacks an RNA-binding domain. In this case, we detect the precipitation of the truncated factor on the resin by Western blot but do not detect mRNA coprecipitated with it, even with nonspecific elution with ExtractRNA. The absence of background precipitation of nonspecific complexes is one way to confirm the high specificity of the Myc-binding resin for these types of experiments.

Furthermore, using a modification of this protocol, it is possible to study the effects of other proteins on the stability of the complex of the mRNA-binding protein of interest with its target mRNA. To this end, mRNA of another protein can be simply coinjected into embryos with the mRNA of mRNA-binding protein of interest. Thus, in our work (Parshina et al., 2020), we studied the effects caused by the cytoskeletal protein Zyxin on the stability of complexes formed by Ybx1 with mRNAs of certain pluripotency genes: *pou 5f3.1*, *pou 5f3.2*, and *pou 5f3.3*.

The example shown in Figure 4 reveals the immunoprecipitation of full-length Myc-tagged Ybx1 in complexes with bound mRNA of pluripotency genes *klf4*, *pou5f3.1*, *pou5f3.2*, *pou5f3.3*, *sox2* and *vent2.2*, as well as the effects of the cytoskeletal protein Zyxin on the stability of these complexes. Figure 4A also shows the results of testing the amount of 6Myc-Ybx1 bound to Myc-binding resin by Western blotting when the compound was expressed in embryos alone and with Zyxin. This test is important because it confirms equal amounts of 6Myc-Ybx1 protein on the resin.

Figure 4B shows the qRT-PCR results of pluripotency gene mRNAs coprecipitated with 6Myc-Ybx1. In this way, we showed in our work that full-length Myc-Ybx1 forms complexes with all *pou5f3* mRNAs, as well as with *vent2.2* and *sox2* mRNAs, but does not bind *klf4* mRNA. The coexpression of Ybx1 with Zyxin has a different effect on the stability of these complexes, namely, the stability decreases for the *pou5f3.1*, *pou5f3.2*, and *pou5f3.3* mRNA but increases for *sox2* and *vent2.2* mRNA.

QUANTIFICATION AND STATISTICAL ANALYSIS

To quantify the results of RIP (RNA immunoprecipitation) we used the approach of Guo et al., 2016: the efficiency of immunoprecipitation is assessed in the form of the IP: input ratio.

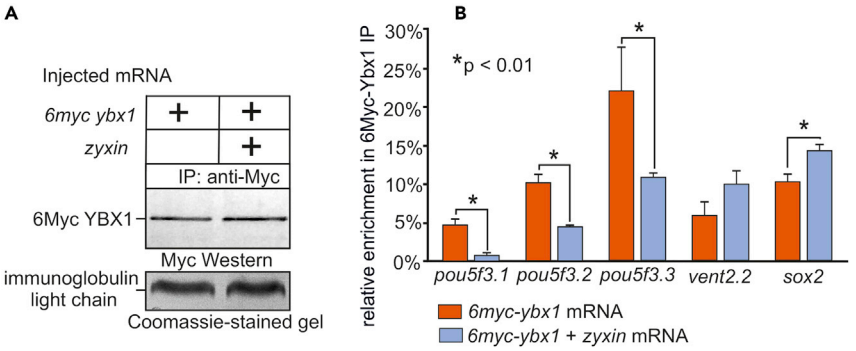

Figure 4. Immunoprecipitation of 6Myc-Ybx1 and RT-qPCR determination of the mRNA of interest precipitated in a complex with 6Myc-Ybx1 (expected outcomes)

(A) An equal amount of 6Myc-Ybx1 protein precipitated on Myc resin from a lysate of embryos microinjected with mRNA encoding *6myc-ybx1* and with a mixture of mRNA encoding *6myc-ybx1* and *zyxin* mRNA. Loading control is shown by the immunoglobulin light chain.

(B) RIP experiments demonstrate the ability of Ybx1 to bind mRNA of pluripotency genes. The addition of *zyxin* mRNA has different effects on the stability of these complexes.

The transcript levels of the housekeeping genes *odc* and *efα* are used for normalization. The error bars indicate the standard deviations derived from three independent experiments. *p<0.01; Student's t-test.

Table 1. EXAMPLE dataset and subsequent calculations

	ybx1 mRNA input	*ybx1+zyxin* mRNA input	*ybx1* mRNA IP	*ybx1+zyxin* MRNA IP	*ybx1* ΔCt	*ybx-1+zyxin* ΔCt	percent total *ybx1*	percent total *ybx1+zyxin*
pou5f3.1	32.4	34.2	33.7	38.2	-1.3	-4	4.79%	0.74%
pou5f3.2	28	29.6	28.2	31	-0.2	-1.4	10.27%	4.47%
pou5f3.3	27.2	29.5	26.3	29.6	0.9	-0.1	22.02%	11.01%
vent2.2	27.1	26.6	27.7	26.8	-0.6	-0.2	7.79%	10.27%
sox2	30.4	30.7	30.7	30.3	-0.3	0.4	9.58%	15.57%
klf4	34.1	35.1	45	45	-10.9	-9.9	0.01%	0.01%

In this approach, the amount of co-precipitated RNA is calculated as a percentage of total input using the following formula: Δ CT (cycle threshold) = CT (input) - CT (IP), percent total = $2^{\Delta CT}$*11.8%, a 100 μL aliquot taken from each of 850 μL of lysate before antibody incubation serves as the input.

The signal from the input samples represented 11.8% of the total RNA used in each RIP. CT values were determined by choosing threshold values in the linear range of each PCR reaction with low variability. We have provided an example dataset to show the calculated values (Table 1).

LIMITATIONS

This protocol can be used for embryos of another species whose development occurs in the external environment, since it allows the introduction of mRNA of the proteins of interest at the beginning of development and the selection of embryos at research stages, for example, for *Danio rerio*, *Strongylocentrotus purpuratus*, *Nematostella vectensis,* C. elegans. When using this protocol, it is important to remember that we are introducing an exogenous labeled factor that can affect many processes in embryonic cells. This protocol is suitable for application to a well-studied factor for which the potential effects of its overexpression are known. In addition, when studying the mRNA repertoire of the investigated factor, it is necessary to take into account the temporal and spatial pattern of the expression of the genes under study, whose transcripts will be studied for complex formation. Taking into account that synthetic mRNA is distributed unevenly between the cells of the embryo during cleavage and that different synthetic RNAs have different diffusion rates and lifetimes, we recommend using this protocol for the early stages of development (no more than 48 h) and a limited number of cleavages (no later than the gastrula stage). In addition, it is always necessary to check the level of the target protein, which is easy to do when using tagged peptides. However, without tags for the immunodetection of proteins in under-studied organisms, it is necessary to obtain specific antibodies.

TROUBLESHOOTING

Problem 1

You received few or inadequate frogs eggs (step 4, Manual egg collection, before you begin)

Potential solution

If the frogs are not producing enough eggs, wait a few hours. Egg quality usually improves a few hours after the first clutch.

Primed frogs are more likely to produce good quality eggs.

Frogs need a two month recovery period between ovulations.

Problem 2

The eggs haven't turned upwards with an animal pole (step 13, In vitro fertilization, before you begin)

Potential solution

If the eggs have not turned over with the animal pole up, then the eggs are not fertilized. This is due to the poor quality of the testis. Motile sperm should be visible under a microscope. If they are not there, then it is better to take the testis from another *X.Laevis* male.

Problem 3

Lower RNA yield than expected in output control (step 10, Immunoprecipitation of Myc-Tagged Ribonucleoprotein Complexes, step-by-step method details).

Potential solution

As noted in before you begin Section, embryonic cell lysates contain many proteolytic enzymes and RNases, so an increased concentration of inhibitors of these enzymes should always be used in lysis and precipitation solutions. For protease inhibitors, no more than 50-fold dilution should be used; for inhibition of RNases, 400 μM of Vanadyl ribonucleoside must be added.

To work with RNA, RNase-free instruments, tips and tubes, gloves, and purified RNase-free water should be used as recommended in this protocol (before you begin).

Problem 4

No detection of target protein in the lysate (step 5, Preparation of lysate from embryonic cells, step-by-step method details).

Potential solution

If the expression of the tagged protein in the lysate of the embryonic cells does not appear as a band on a Western blot, or the band does not correspond to the expected molecular weight of the protein, then it is necessary to

1-sequence the vector that was used for the in vitro transcription and check for reading frame errors, stop codons, and the presence of a sequence for the synthesis of poly-A, Kozak consensus, etc.

2-check for the presence of restriction enzymes used to linearize the vector in frame.

3-ensure that the template for RNA synthesis contains a poly A coding sequence.

4-use a cap analog for transcription.

Problem 5

No protein precipitation on the affinity gel. (step 14, Proteolysis and RNA isolation, step-by-step method details).

Potential solution

If there are no problems with the expression of the target protein, then the lack of precipitate may be due to its interaction with antibodies on the affinity gel. We observed this effect when using specific antibodies to endogenous and exogenous proteins, but this is not the case when using tagged proteins with commercially available affinity gels. However, tag size and location (N or C terminus) can have an impact on protein folding and/or function, and should be taken into consideration. Usually small tag peptides do not interfere with the function, but sometimes they can be removed by protein maturation (processing). Therefore, we highly recommend the use of tagged proteins for the RNA precipitation experiments.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for and reagents should be directed to and will be fulfilled by the lead contact, Andrey Zaraisky (azaraisky@yahoo.com).

Materials availability

No materials were generated in this study.

Data and code availability

No data or code was generated in this study.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2021.100552.

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AUTHOR CONTRIBUTIONS

Conceptualization, A.G.Z. and N.Y.M.; methodology, N.Y.M., E.A.P., and A.G.Z.; investigation, E.A.P., N.Y.M., and A.G.Z.; writing original draft, N.Y.M. and E.A.P.; writing review and editing, N.Y.M. and A.G.Z.; funding acquisition, A.G.Z. and N.Y.M.

DECLARATION OF INTERESTS

The authors declare no competing interests

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